An investigation into the expression and role of cannabinoid receptors in T lymphocytes

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An investigation into the expression and role of cannabinoid receptors in T lymphocytes

A thesis submitted by

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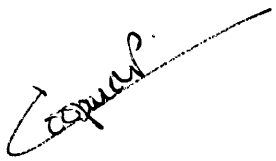

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Abstract

Cannabinoids have long been proposed to affect the immune system, especially as one of the cannabinoid receptors, the cannabinoid receptor-2 (CB_2R) has been found almost exclusively on immune cells. Here, using human *in vitro* activated peripheral blood-derived T lymphocytes (PBLs) the long-term changes in cannabinoid receptor protein expression following cellular activation and the effects of cannabinoids on migration and proliferation were investigated. CB_2R protein expression is upregulated in T lymphocytes following activation by the superantigen Staphylococcal enterotoxin B (SEB). This high level of expression peaks around day 5 of culture and is maintained for several days before being downregulated. Activating the cells using CD3/CD28-coated beads results in a longer-lasting upregulation of receptor protein expression. In addition, PBLs also express an 83kD form of the cannabinoid receptor-1 (CB_1R) but not the 53kD protein. Although the receptors were found to be coupled to downstream biochemical effectors (as assessed by the phosphorylation of the extracellular signal-regulated kinase 1/2), neither the endogenous cannabinoid 2-arachidonoylglycerol nor the CB_2R-selective synthetic agonist JWH-133 induced chemotaxis in day 5 or day 12 SEB-activated PBLs, when receptor expression was at its highest. However, they both inhibited CXCL12-induced chemotaxis of day 5 and 12 PBLs. In contrast, JWH-133 increased IL-2-induced proliferation of day 5, but not day 11, PBLs suggesting that changes in CB_2R expression affects some but not all T lymphocyte functions. The data presented here corroborates studies which suggest a modulatory role for cannabinoids in the immune response.

Abbreviations

2-AG	2-arachidonoylglycerol
5-HT	5-hyrdoxytryptamine
AA	arachidonic acid
Abn-cannabidiol	abnormal cannabidiol
AC	adenylyl cyclase
ACEA	arachidonyl-2'-chloroethylamide
ACPA	arachidonoylcyclopropylamide
ADF	actin-depolymerising factor
AEA	*N*-arachidonoylethanolamide, anandamide
AMT	anandamide membrane transporter
AP-1	activator protein-1
APC	antigen-presenting cell
aPKC	atypical PKC
Arp2/3	actin-related protein 2/3
BSA	bovine serum albumin
C5a	activated complement component 5
CaM	calmodulin
CaMK	calmodulin-dependent kinase
cAMP	cyclic adenosine mono-phosphate
CB_1R	cannabinoid receptor-1
CB_2R	cannabinoid receptor-2
CD	cluster of differentiation
CNS	central nervous system
COX-2	cyclooxygenase-2
cPKC	conventional PKC
CRE	cAMP response element
CRIP	cannabinoid receptor interacting protein
CTLA-4	cytotoxin T lymphocyte antigen-4
CXC	CXCL12
DAG	diacylglycerol
DAGL	DAG lipase
DMSO	dimethyl sulphoxide
Ena/VASP	enabled/vasodilator-stimulated phosphoprotein
Epac	exchange factor directly activated by cAMP
ERK	extracellular signal-regulated kinase
FAAH	fatty acid amide hydrolase
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	foetal bovine serum
fMLP	formyl-metionyl-leucine-phenylalanine
FRNK	focal adhesion kinase-related non-kinase
G-actin	globular actin
GM-CSF	granulocyte macrophage-colony stimulating factor
Gö	Gö6976
GPCR	G-protein-coupled receptor
Grb2	growth factor receptor-bound 2

GRK	G-protein-coupled receptor kinase
HA	hyaluronan
HETEE	hydroxyeicosatetraenoylethanolamide
HETE-G	hydroxyeicosatetraenoyl-glycerol ester
HPETEE	hydroperoxyeicosatetraenoylethanolamide
HPETE-G	hydroperoxyeicosatetraenoyl-glycerol ester
IBD	inflammatory bowel disease
ICAM-1	intracellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IP	immunoprecipitation
IP_3	inositol 1,4,5 triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
IκB	inhibitor of NFκB
JNK	c-Jun N-terminal kinase
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LFA-1	leukocyte function associated antigen-1
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
LTB_4	leukotriene B4
LY	LY294002
MAFP	methyl arachidonoyl fluorophosphonate
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MEK	MAPK/ERK kinase
MEKK	MEK kinase
Met-AEA	methanandamide
MHC	major histocompatability complex
MKK	mitogen-activated protein kinase kinase
MLC	myosin light-chain
MLCK	MLC kinase
MLK	mixed lineage kinase
MTOC	microtubule-organising centre
NAAA	*N*-acylethanolamine-hydrolysing acid amidase
NADA	*N*-arachidonoyl dopamine
NAE	*N*-acylethanolamine
NAPE	*N*-arachidonoyl phosphatidylethanolamine
NEMO	NFκB essential modulator
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor for immunoglobulin κ chain in B cells
NK	natural killer
nPKC	novel PKC
PAK	p21 activated kinase
PBL	peripheral blood-derived T lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline

PD	PD98059
PDE	phosphodiesterase
PDK1	3'phosphoinositide-dependent kinase-1
PE	phosphatidylethanolamine
PEA	*N*-palmitoylethanolamine
PGx-G	prostaglandin x-glycerol ester
PGx-EA	prostaglandin x-ethanolamide
PH	pleckstrin homology
PHA	phytohemagglutinin
PI	phosphoinositide
PI3K	phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 13-myristate 12-acetate
PMS	phenazine methosulfate
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PtdIns	phosphatidylinositol
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PTX	pertussis toxin
Pyk2	proline-rich tyrosine kinase-2
RACK	receptor for activated PKC
RIP	receptor-interacting protein
RO	Ro-32-0432
ROCK	Rho kinase
Rott	rottlerin
RTK	receptor tyrosine kinase
S1P	sphingosine-1-phosphate
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B
Shc	Src homology and collagen
SOS	son of sevenless
TBS	tris-buffered saline
TBST	TBS-Tween
TCR	T cell antigen receptor
TGF	transforming growth factor
THC	tetrahydrocannabinol
TM	transmembrane
TNFR1	TNFα receptor 1
TNFα	tumour necrosis factor α
TRADD	TNF-receptor associated death domain protein
TxA_2-EA	thromboxane A_2-ethanolamide
TxA_2-G	thromboxane A_2-glycerol ester
VR1	vanilloid receptor type 1

WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin homologous protein
WCL	whole cell lysate
ZAP-70	70kD TCR ζ-chain-associated protein kinase

Chapter 1: Introduction

Marijuana, or cannabis, from the plant *Cannabis sativa* L., has been used medicinally for thousands of years for a number of complaints such as gastrointestinal disorders and rheumatic pain. Yet its use therapeutically remains controversial due to the psychoactive effects that are associated with it. Much of the current research in the field is aimed at understanding how cannabinoids exert their effects on physiological properties such as memory, cardiovascular function, cognition, pain, reproduction, motor control and immune function, in order to improve our understanding of how they could be better exploited therapeutically (Carlini, 2004, Pertwee, 2006).

Many initial studies suggested that cannabinoids are immunosuppressive, although more recent data has shown that they can exert both pro and anti-inflammatory actions and thus they are now commonly referred to as being immunomodulatory (Croxford & Yamamura, 2005). One of the targets of cannabinoids in the immune system is T lymphocytes. Although some research regarding the effects of cannabinoids on T lymphocyte functions has been carried out their role still remains unclear, especially as there is much conflicting data published. Thus, this study was undertaken to further investigate the role of cannabinoids in T lymphocytes, in particular with respect to migration and proliferation.

1.1 The cannabinoid system

1.1.1 Background

The field of cannabinoid research really began in the 1960s when the main active ingredient of *Cannabis sativa* L., Δ^9-tetrahydrocannabinol (Δ^9-THC), was isolated (Gaoni & Mechoulam, 1964). It is this natural plant derivative, as well as the many others like it such as cannabidiol and cannabinol, to which the term cannabinoid originally referred. The isolation of Δ^9-THC led to the development of a number of synthetic cannabinoids. Initially, it was believed that these lipophilic compounds were acting by disrupting cellular membranes until the existence of specific cannabinoid binding sites in the rat brain was established (Devane *et al.*, 1988). Two distinct cannabinoid receptors were rapidly cloned,

the cannabinoid receptor-1 (CB_1R; Matsuda *et al.*, 1990, Gerard *et al.*, 1991) which is located mainly in the central nervous system (CNS) and the cannabinoid receptor-2 (CB_2R; Munro *et al.*, 1993) which is expressed in cells of the immune system. The discovery of these receptors led to the search for an endogenous ligand and so far several possible 'endocannabinoids' have been identified. *N*-arachidonoylethanolamide (AEA, anandamide; Devane *et al.*, 1992) and 2-arachidonoylglyercol (2-AG; Mechoulam *et al.*, 1995) have been the most widely studied. Thus the term cannabinoid now generally refers to all those compounds which activate the cannabinoid receptors and includes the natural plant derived, synthetic and endogenous compounds. The 'cannabinoid system' comprises not only the cannabinoid receptor and the endocannabinoids, but also a putative transporter and the enzymes involved in the inactivation of the endocannabinoids. The term 'cannabinergic ligand' is a general one used to describe those compounds that act on any of the proteins within the cannabinoid system, regardless of their chemical classification or type of resultant pharmacological activity.

1.1.2 The cannabinergic ligands

1.1.2.1 AEA & 2-AG

1.1.2.1.1 Synthesis

AEA belongs to a large family of lipid structures known collectively as the *N*-acylethanolamines (NAEs), many of which are cannabinoid receptor inactive. AEA, like all NAEs can be produced via a two step process involving the production of *N*-arachidonoyl phosphatidylethanolamine (NAPE) which is then hydrolysed to AEA and phosphatidic acid (Fig. 1.1; Sugiura *et al.*, 2002, Bisogno *et al.*, 2005). The mechanisms involved in the regulation of AEA biosynthesis are still unclear although AEA appears to be synthesised and immediately released rather than stored in vesicles (Goutopoulos & Makriyannis, 2002, Sugiura *et al.*, 2002, Bisogno *et al.*, 2005). AEA is thought to be locally produced to act on the same or neighbouring cells as an autocrine or paracrine mediator. Levels are thought to lie in the low pmol/g range for most tissues

although these can change in pathological conditions (Goutopoulos & Makriyannis, 2002, Sugiura *et al.*, 2002).

2-AG belongs to the family of monoacylglycerols and is potentially produced by several different pathways (Sugiura *et al.*, 2002, Bisogno *et al.*, 2005). The main one studied has been the conversion of 1,2-diacylglycerol (DAG) to 2-AG by a DAG lipase (DAGL; Fig. 1.1). DAG can be generated from membrane phosphoinositides (PIs) by phospholipase C (PLC). Like AEA, 2-AG is also synthesised and released on demand but levels of 2-AG generally exceed those of AEA, tending to be in the low nmol/g range in different tissue types (Sugiura *et al.*, 2002).

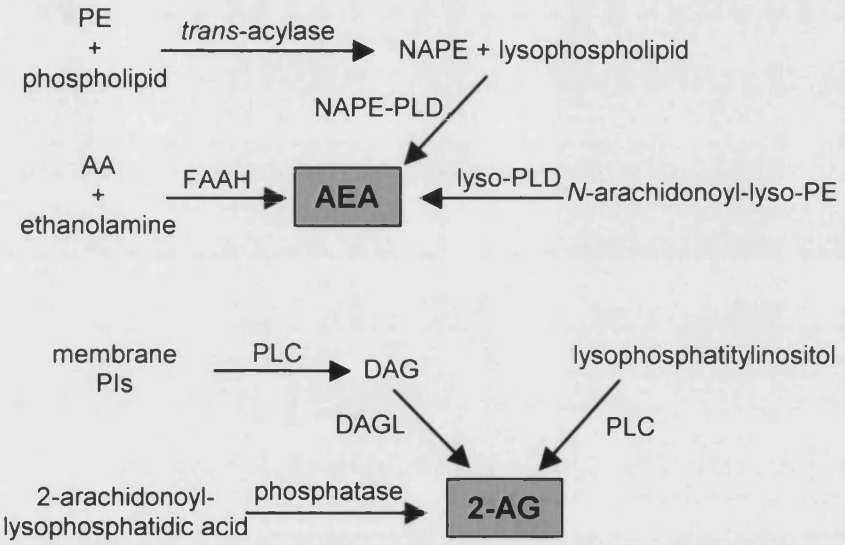


Figure 1.1: **AEA and 2-AG synthesis.** An outline of some of the AEA and 2-AG biosynthesis pathways described to date. AEA is believed to be mainly generated from NAPE by a specific phospholipase D (PLD) selective for NAPEs (NAPE-PLD; Sugiura *et al.*, 2002, Bisogno *et al.*, 2005). Although the physiological relevance is unclear, it is possible that AEA is also generated from free arachidonic acid (AA) and ethanolamine by fatty acid amide hydrolase (FAAH; Sugiura *et al.*, 2002). Finally, it may also be generated from *N*-arachidonoyl-lyso-PE which is a product of phospholipase A_2 action on NAPE (Sugiura *et al.*, 2002, Bisogno *et al.*, 2005). 2-AG also has several different possible biosynthetic pathways (Sugiura *et al.*, 2002, Bisogno *et al.*, 2005). The main one is its generation from diacylglycerol (DAG) by two *sn*-1 DAGLs, DAGLα and γ. DAG itself can be generated from several different sources but only one is shown. 2-AG may also be generated from 2-arachidonoyl lysophosphatidic acid or from lysophosphatidylinositol. *Abbreviations*: PE, phosphatidylethanolamine.

Questions on how or whether these endocannabinoids are generated over each other or other members of their families still remain. The enzymes involved in

AEA and 2-AG synthesis, such as the NAPE-PLD or DAGL are not selectively involved in the generation of AEA or 2-AG but rather NAEs and monoacylglycerols in general (Sugiura *et al.*, 2002, Bisogno *et al.*, 2005). In some instances AEA and 2-AG levels can be independently modified whereas in other cases levels of both are increased or decreased (Pertwee & Ross, 2002). Many of the cannabinoid receptor inactive NAEs and monoacylglycerols can elicit cannabinoid-like effects and it has been hypothesised that they may be acting through an 'entourage effect' (Schmid *et al.*, 2002). That is to say that they interfere with AEA and 2-AG transport and inactivation, thereby enhancing the cannabimimetic activity of these endocannabinoids. However, this has only been reported *in vitro* and whether the entourage effect hold true *in vivo* remains controversial as topical administration of PEA in mice resulted in decreasing AEA levels in skin (Lo Verme *et al.*, 2005a). Furthermore, some NAEs, such as *N*-palmitoylethanolamine (PEA), may elicit actions through specific non-cannabinoid receptors (Lo Verme *et al.*, 2005b).

1.1.2.1.2 Transport

Once released AEA and 2-AG are rapidly removed from the extracellular space, limiting their pharmacological actions. Several different mechanisms for AEA transport have been proposed. A putative anandamide membrane transporter (AMT) has been pharmacologically characterised after it was shown that uptake of AEA is facilitated by a saturable, temperature-dependent, Na^+-independent and selective carrier protein in several different cell types (Bisogno *et al.*, 2001, Bisogno *et al.*, 2005). However, thus far the protein remains uncharacterised at the molecular level. It has also been reported that AEA uptake may simply be due to diffusion which is facilitated by the intracellular breakdown of AEA by FAAH, one of the key enzymes involved in its metabolism, which shifts the AEA concentration gradient such that it favours diffusion into the cell without the involvement of a carrier protein (Glaser *et al.*, 2003). Furthermore, it has also been suggested that the two mechanisms may act in concert (Day *et al.*, 2001, Deutsch *et al.*, 2001) and that FAAH alone is insufficient to cause accumulation, or that it may play no role at all (Bisogno *et al.*, 2005, Moore *et al.*, 2005). It has also been proposed that AEA is sequestered either in a lipid

compartment or by binding a specific protein once it enters the cell, hence lowering the concentration of free AEA to favour AEA accumulation into the cell (Hillard & Jarrahian, 2003). Finally, AEA uptake may take place via a caveolae-related endocytic process that targets AEA to intracellular compartments where it can then be metabolised (McFarland *et al.*, 2004). Not all cells accumulate AEA to the same degree and therefore it is likely that the uptake mechanism is cell-type specific and that each process may play a role. Equally, studies have so far been inconclusive as to whether a 2-AG transporter exists and if it does, whether it is the same protein as the AMT (Di Marzo *et al.*, 1999, Bisogno *et al.*, 2001, Hajos *et al.*, 2004, Hermann *et al.*, 2006).

1.1.2.1.3 Inactivation

Once inside the cell both endocannabinoids are rapidly degraded and it is possible that some of the responses attributed to AEA and 2-AG may in fact be caused by their metabolites (Pertwee & Ross, 2002). The main metabolic pathways are summarised in Fig. 1.2. FAAH is the main enzyme involved in AEA degradation (Cravatt & Lichtman, 2002). This fairly widely expressed, membrane-bound, enzyme is capable of hydrolysing a wide range of NAEs to their fatty acids and belongs to the amidase signature family of enzymes (Cravatt & Lichtman, 2002). It is also capable of hydrolysing 2-AG (Goparaju *et al.*, 1998). AEA, but not 2-AG, is also hydrolysed by *N*-acylethanolamine-hydrolysing acid amidase (NAAA), although AEA is a poorer substrate than some other NAEs such as PEA (Ueda *et al.*, 1999, Ueda *et al.*, 2001). It belongs to the choloylglycine hydrolase family of enzymes and is closely related to acid ceramidase although it has no ceramidase activity (Tsuboi *et al.*, 2005). It is widely expressed, although some differences between species have been noted (Sun *et al.*, 2005). It has also been suggested that certain cytochrome P450s may metabolise AEA (Kozak & Marnett, 2002). Although the physiological relevance of this pathway is largely unknown it has been shown that AEA can indirectly activate TRPV4, a cation channel, via a mechanism involving its metabolism to epoxyeicosatetranoic acids by cytochrome P450 (Watanabe *et al.*, 2003).

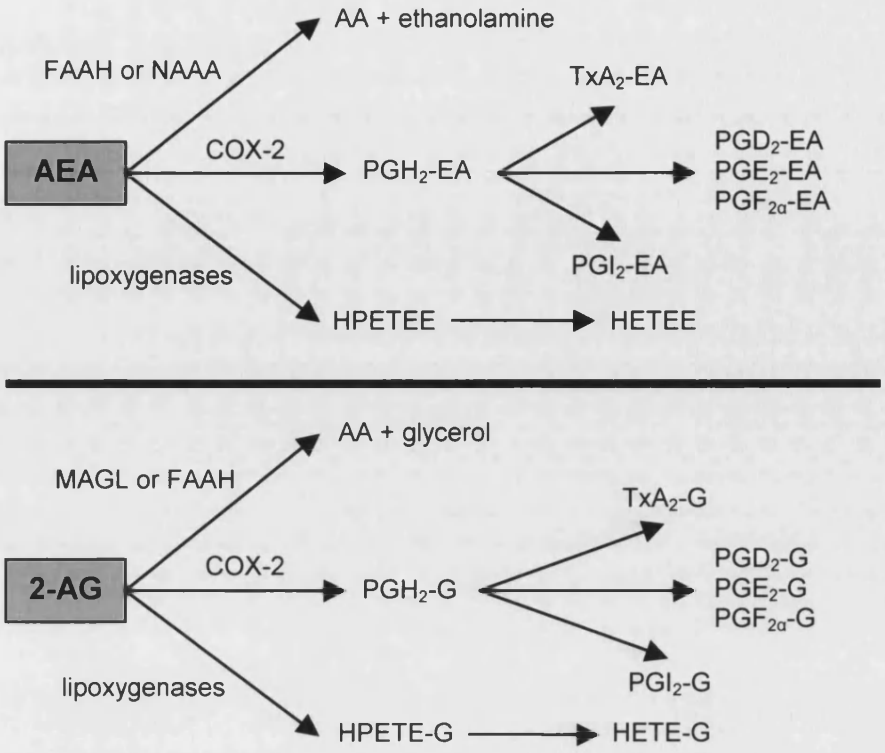


Figure 1.2: **AEA and 2-AG metabolism.** An outline of some of the pathways for AEA and 2-AG inactivation. These pathways may produce a range of metabolites that may have their own pharmacological effects. As well as being hydrolysed both AEA and 2-AG can be metabolised by cyclooxygenase-2 (COX-2) and lipoxygenases and it has been shown, mainly through in vitro studies, that the resulting prostaglandin H_2-ethanolamide (PGH_2-EA) and-glycerol ester (PGH_2-G) can then be converted into the corresponding prostaglandin-ethanolamides (prostamides) or –glycerol esters, thromboxane A_2-ethanolamide (TxA_2-EA) or –glycerol ester (TxA_2-G), and hydroperoxyeicosatetraenoic acid related products. *Abbreviations*: HETEE, hydroxy-eicosatetraenoylethanolamide; HETE-G, hydroxy-eicosatetraenoyl-glycerol ester; HPETEE, hydroperoxyeicosatetraenoylethanolamide; HPETE-G, hydroperoxyeicosa-tetraenoyl-glycerol ester; MAGL, monoacylglycerol lipase.

Both AEA and 2-AG are also substrates for COX-2 (Kozak *et al.*, 2002a) and some lipoxygenases (Edgemond *et al.*, 1998, Moody *et al.*, 2001, Kozak *et al.*, 2002b), producing a number of novel prostanoid and hydroxyeicosatetraenoic acid related products (Fig. 1.2). It is largely unknown what physiological relevance these metabolites have and how they may act but a few studies have suggested that they could represent novel signalling molecules. For instance, undefined lipoxygenase AEA products may act via the vanilloid receptor 1 (VR1) in the guinea pig bronchus (Craib *et al.*, 2001), 15-HETE-G can activate peroxisome proliferator-activated receptor (PPAR)-α (Kozak *et al.*, 2002b) and PGE_2-G has been shown to mobilise calcium in the murine macrophage cell line,

RAW264.7 (Nirodi *et al.*, 2004). It is important to note that the relevance of these metabolites may change in pathological states.

2-AG can also be hydrolysed by MAGL (Fig. 1.2). This widely expressed enzyme can degrade a range of unsaturated monoacylglycerols but not AEA (Dinh *et al.*, 2002, Goparaju *et al.*, 1999, Karlsson *et al.*, 1997). In addition, 2-AG could enter a number of lipid metabolic pathways to produce DAG or phosphatidic acid (Schmid *et al.*, 2002) and it is rapidly converted to 1(3)-AG by acyl migration (Rouzer *et al.*, 2002).

1.1.2.1.4 Mechanism of action

AEA and 2-AG appear to be produced on demand rather than stored and are thought to act as autocrine or paracrine mediators (Giuffrida *et al.*, 2001). They couple to number of intracellular pathways, such as activation of mitogen-activated protein kinases (MAPKs), inhibition of cyclic adenosine mono-phosphate (cAMP) accumulation and modulation of intracellular Ca^{2+} concentrations to exert their various actions. They can bind to and activate two cannabinoid receptors, the CB_1R and CB_2R. Studies have shown that AEA is a partial agonist at both the CB_1R and CB_2R whilst 2-AG acts as a full agonist at both receptors (Sugiura *et al.*, 1999, Sugiura *et al.*, 2000, Howlett *et al.*, 2002). Several additional cannabinoid receptors have also been described as will be discussed later and may be involved in some of the actions of AEA and 2-AG (Howlett *et al.*, 2002, Wiley & Martin, 2002, Begg *et al.*, 2005, Demuth & Molleman, 2006).

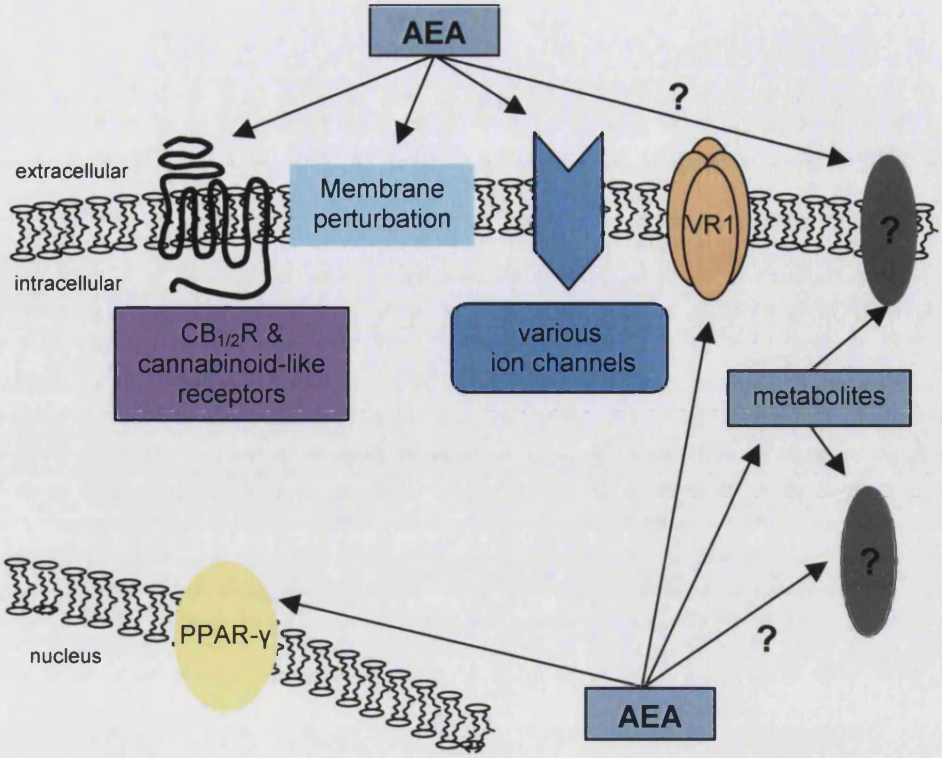


Figure 1.3: **Schematic showing the various receptors and ion channels that the endocannabinoid AEA can interact with.**

As shown in Fig. 1.3 there are also additional ways in which AEA can exert its actions. For instance, AEA has been shown to directly activate the VR1, a non-selective cation channel (Smart *et al.*, 2000, Ross, 2003). It has low intrinsic efficacy at the VR1 and acts as a partial agonist in tissues with low receptor reserve and a full agonist in tissues with high receptor reserve (Ross, 2003). 2-AG has been shown to be a poor agonist for the VR1 (Zygmunt *et al.*, 1999, Golech *et al.*, 2004). AEA can also directly interact with a number of neurotransmitter-gated ion channels such as NMDA, 5-hydroxytryptamine (5-HT, serotonin) and α7-nicotinic acetylcholine-receptors *in vitro*, although the *in vivo* relevance of this is still not known (van der Stelt & Di Marzo, 2005). It has also been shown to bind the nuclear receptor PPAR-γ and induce PPAR-γ-dependent transcriptional activity (Bouaboula *et al.*, 2005). Similarly, 2-AG has also been shown to activate PPAR-γ (Bouaboula *et al.*, 2005, Burstein, 2005, Rockwell *et al.*, 2006). It is also possible that membrane perturbation, which was originally thought to be the mechanism of action of cannabinoids before the receptors were discovered, may still play a role in the actions of AEA and 2-AG

(Bloom *et al.*, 1997, Ambrosi *et al.*, 2005). It was shown in one study that cannabinoids do alter membrane lipid ordering but it was not the primary mechanism of action as the pharmacological potency did not correlate well with their potency for membrane perturbation (Bloom *et al.*, 1997). Recently, a role for lipid rafts in some AEA actions has also been suggested as membrane cholesterol depletion, which disrupts raft integrity, has been shown to affect CB_1R binding and subsequently signal transduction as well as cannabinoid receptor and VR1-independent signalling (Sarker & Maruyama, 2003, Bari *et al.*, 2005a, Bari *et al.*, 2005b). As already mentioned, it is also possible that some of the AEA and 2-AG actions are due to their metabolites having pharmacological actions (Craib *et al.*, 2001, Kozak *et al.*, 2002b, Pertwee & Ross, 2002, Nirodi *et al.*, 2004) and there may still exist more unidentified mechanisms by which AEA and 2-AG may exert some of their actions.

1.1.2.2 Other endocannabinoids

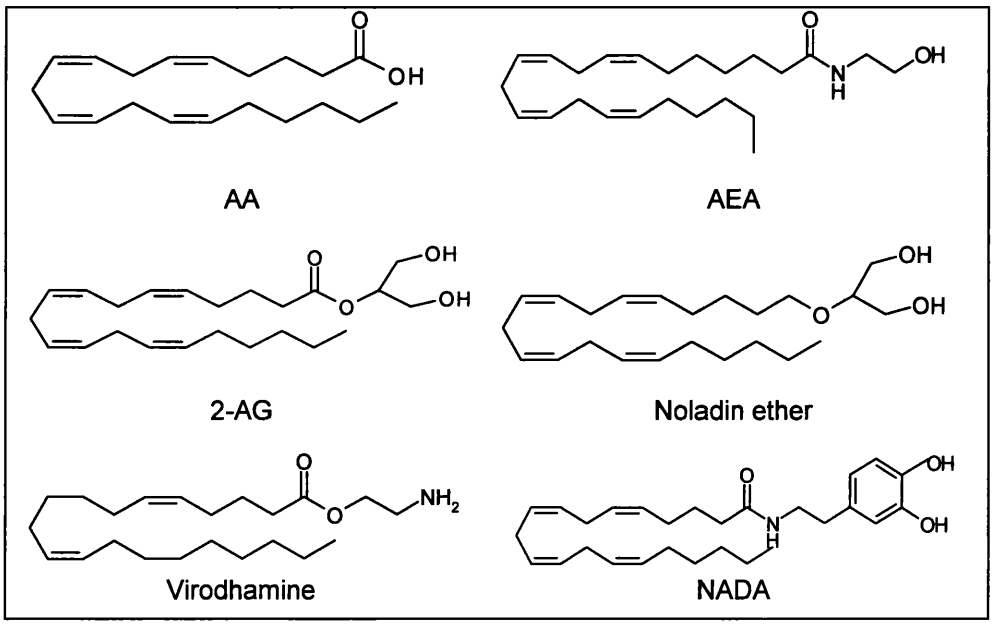


Figure 1.4: **Structures of AA and some of the best characterised endocannabinoids.**

In addition to AEA and 2-AG there are numerous other endocannabinoids (Bradshaw & Walker, 2005) with varying affinities for the cannabinoid receptors. Of these, 2-arachidonoyl glycerol ether (noladin ether), *O*-

arachidonoyl ethanolamide (virodhamine) and *N*-arachidonoyl dopamine (NADA) are among the best characterised. Their structures, along with those of AEA and 2-AG, are shown in Fig. 1.4.

1.1.2.2.1 Noladin ether

Noladin ether was originally developed synthetically as a stable analogue of 2-AG (Sugiura *et al.*, 1999). Its classification as an endocannabinoid is somewhat controversial as, although it has been shown to be naturally occurring in porcine (Hanus *et al.*, 2001) and rat (Fezza *et al.*, 2002) brain, Oka *et al* (2003) failed to isolate appreciable amounts from rat, mouse, hamster, guinea pig and pig brain. Little is known about its biosynthesis and it is suggested that as its ether bond cannot be hydrolysed enzymatically, esterification is likely to be important in its inactivation (De Petrocellis *et al.*, 2004). It has been reported that, like AEA and 2-AG, it is taken up into cells by a similar or perhaps identical membrane transporter, and that it is inactivated in a time, concentration and temperature-dependent manner (Hanus *et al.*, 2001). Initially, it was believed to be a CB_1R-selective agonist (Hanus *et al.*, 2001) with a much higher affinity for the CB_1R than CB_2R. However, a recent report suggests that noladin ether does in fact act as a full agonist at the human CB_2R with high nanomolar affinity (Shoemaker *et al.*, 2005a). It may also exert CB_1R and CB_2R-independent effects (Duncan *et al.*, 2004).

1.1.2.2.2 Virodhamine

Virodhamine, an ester-linked isomer of AEA, acts as a full agonist at the CB_2R but is classified as a CB_1R antagonist/inverse agonist (Porter *et al.*, 2002, Steffens *et al.*, 2005a). It was isolated from rat brain and human hippocampus at concentrations similar to AEA but was expressed at higher concentrations in rat peripheral tissues (Porter *et al.*, 2002). It is not yet fully known how virodhamine is produced, stored or degraded although it can inhibit AEA transport, suggesting that it has the same reuptake mechanism as AEA (Porter *et al.*, 2002) and it is likely to be degraded by FAAH (Steffens *et al.*, 2005a).

1.1.2.2.3 NADA

NADA, like AEA, is both an endocannabinoid and an endovanilloid (Bisogno *et al.*, 2000, Huang *et al.*, 2002). It has been found in rat and bovine nervous tissue (Huang *et al.*, 2002) and has been shown to be an agonist at both the CB_1R (Bisogno *et al.*, 2000) and the VR1 (Huang *et al.*, 2002, Toth *et al.*, 2003) although higher concentrations are required to activate the CB_1R than the VR1. As for AEA, its activity at the VR1 is enhanced by protein kinase C (PKC) phosphorylation (Premkumar *et al.*, 2004). CB_1R and VR1-independent effects have been reported, suggesting NADA may act on additional targets (Sancho *et al.*, 2004, Sancho *et al.*, 2005). Currently very little is known about its biosynthesis or breakdown but it appears that there may be more than one route of synthesis (Huang *et al.*, 2002, De Petrocellis *et al.*, 2004).

1.1.2.3 Other cannabinergic ligands

As well as the endocannabinoids, there are numerous other cannabinergic ligands and it would simply not be possible to mention them all here. Table 1 lists a few of the commonly used cannabinoid receptor agonists and antagonists as well as their equilibrium dissociation constants at each receptor whilst Fig. 1.5 illustrates some of their structures. For more in depth reviews please refer to Howlett *et al*, 2002 and Palmer *et al*, 2000.

Currently there are 70 known natural cannabinoids and Δ^9-THC, cannabidiol and cannabinol are the most prevalent of these. They have also been the most widely studied. Δ^9-THC is the most psychologically active constituent of cannabis and acts as a partial agonist at both the CB_1R and CB_2R (Elsohly & Slade, 2005). Δ^9-tetrahydrocannabivarin is an example of a natural CB_1R and CB_2R antagonist (Thomas *et al.*, 2005). A wide variety of synthetic cannabinoid receptor agonists and antagonists have also been generated. One of the most commonly used is CP55,940, a non-specific, high affinity cannabinoid receptor agonist. Its tritiated analogue was used in the study that identified the existence of cannabinoid binding sites in rat brain (Devane *et al.*, 1988). Other non-selective, highly potent agonists include HU210 and WIN55,212-2. CB_1R and CB_2R-selective

agonists have also been produced. For instance, arachidonoylcyclopropylamide (ACPA) and arachidonyl-2'-chloroethylamide (ACEA) are commonly used as CB_1R-selective agonists whilst JWH-133 and JWH-015 are examples of CB_2R-selective agonists. Methanandamide (met-AEA) has also been generated as a stable analogue of AEA and is somewhat selective for the CB_1R. Sanofi created the two CB_1R and CB_2R antagonists SR141716A and SR144528, respectively. In addition, AM251, a CB_1R, and AM630, a CB_2R-selective antagonist are also commonly used. Each of these has also been reported to behave as an inverse agonist (Howlett *et al.*, 2002, Palmer *et al.*, 2002).

Ligand	**Agonist/Antagonist**		**Ki (nM)**	
	CB_1R	CB_2R	CB_1R	CB_2R
2-AG	A	A	472	1400
ACPA	A		2.2	715
AEA	PA	PA	89	371
AM251	AN/IA		7.5	2290
AM630		AN/IA	5152	31.2
CP55,940	A	A	0.58	0.69
HU210	A	A	0.73	0.22
JWH-133		A	677	3.4
met-AEA	A		17.9	868
SR141716A	AN/IA		5.6	>1000
SR144528		AN/IA	437	0.60
WIN55,212-2	A	A	1.89	0.28
Δ^9-THC	PA	PA	53.3	75.3

Table 1: **Examples of ligands that interact with the CB_1R and CB_2R.** The Ki values of the ligands for the *in vitro* displacement of [^{3}H]CP55,940, [^{3}H]WIN55,212-2 or [^{3}H]HU243 from the CB_1R and CB_2R are given as well as whether they have been found to act as an agonist (A), partial agonist (PA), antagonist (AN) or inverse agonist (IA) indicated. The Ki values quoted are taken from Howlett *et al.*, 2002 and Palmer *et al.*, 2002.

The cannabinergic ligands also include inhibitors of FAAH and MAGL and the putative AMT. Phenylmethylsulphonyl fluoride (PMSF) was the first FAAH inhibitor but since then compounds with much greater potency have become available. For instance, methyl arachidonoyl fluorophosphonate (MAFP) is a

potent, irreversible inhibitor of FAAH and can also inhibit MAGL (De Petrocellis *et al.*, 1997, Goparaju *et al.*, 1999, Saario *et al.*, 2004). The most notable of the transport inhibitors is *N*-(4-hydroxyphenyl) arachidonylamide (AM404) which has been reported to inhibit AEA uptake and therefore also increase plasma levels of AEA (Howlett *et al.*, 2002, Palmer *et al.*, 2002).

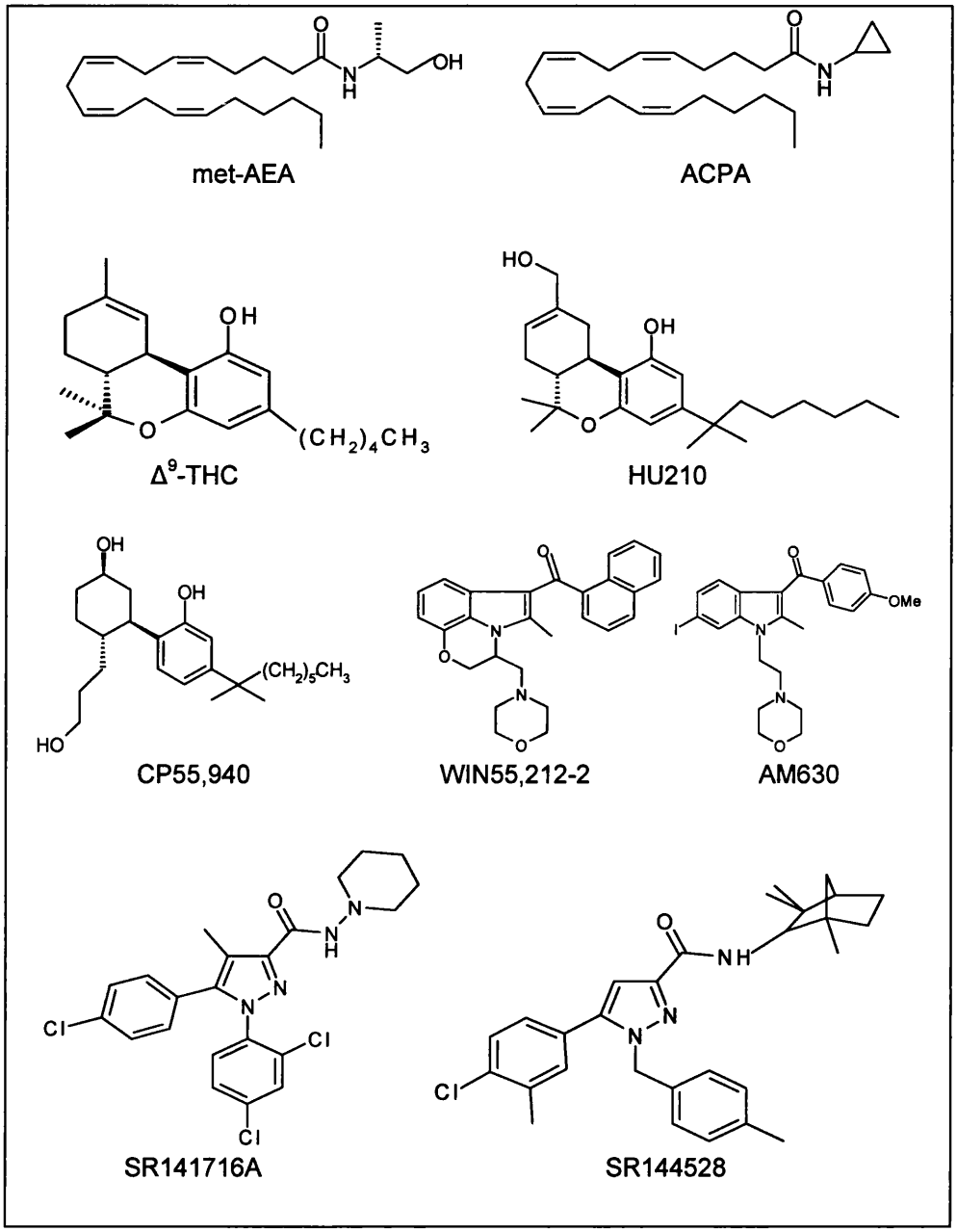


Figure 1.5: **Structures of some of the cannabinergic ligands.**

The list of cannabinergic ligands continues to expand as more synthetic ligands are created in order to assess the role of the individual components of the cannabinoid system in some of the cannabinoid effects. In addition, it has also

been recently reported that *Echinacea* alkylamides upregulate tumour necrosis factor α (TNFα) mRNA in primary human monocytes via the CB_2R as this effect could be inhibited by SR144528 (Gertsch *et al.*, 2004). Although specific binding or interaction of these alkylamides with the CB_2R has not been shown, it does suggest that additional natural cannabinergic ligands may exist.

1.1.3 Receptors

1.1.3.1 CB_1R and CB_2R

The CB_1R was originally cloned from a rat cerebral cortex cDNA library (Matsuda *et al.*, 1990) and cloning of the human CB_1R soon followed (Gerard *et al.*, 1991). The human CB_1R is a 472 amino acid protein with a predicted molecular weight of 53kD (Gerard *et al.*, 1991). Initially it was only detected in the brain, hence it was considered to be the 'central' cannabinoid receptor, but it is now know that it is also located in a number of peripheral tissues, including testes, heart, lung and bone marrow (Howlett *et al.*, 2002). CB_1R mRNA has also been found in immune cells, albeit at much lower levels than in the brain (Bouaboula *et al.*, 1993). It is believed to be involved in a number of cannabinoid actions such as the psychoactive effects (Iversen, 2003), gastric motitility (Izzo *et al.*, 2001) and regulation of food intake (Di Marzo & Matias, 2005).

Soon after the initial discovery of the human CB_1R (Gerard *et al.*, 1991), the existence of an amino terminal splice variant of the human CB_1R, termed CB1A was described (Shire *et al.*, 1995). Although it is expressed in much lower amounts than the CB_1R, CB1A mRNA expression pattern seems to mimic that of the CB_1R. More recently, an additional human CB_1R splice variant was described and termed CB1B (Ryberg *et al.*, 2005). Again, the CB1B differs from the CB_1R and CB1A in its amino terminal sequence and is expressed in a number of tissues. Both splice variants were found to exhibit altered ligand binding and activation properties compared to the CB_1R when all three were transfected into HEK293 cells (Ryberg *et al.*, 2005).

The CB_2R was first called the 'peripheral' cannabioid receptor owing to its discovery in the human promyelocytic cell line HL-60 and macrophages (Munro *et al.*, 1993). It shares 44% amino acid identity with the CB_1R (Munro *et al.*, 1993) and is smaller in size as it has a predicted molecular mass of only 39kD. Its expression pattern in much more restricted than that of the CB_1R, with its mRNA being found largely in a variety of immune subpopulations (Galiegue *et al.*, 1995). However, more recently it has become apparent that it may also be expressed in the CNS, for instance, in oligodendrocytes, cerebellar neurons and the brainstem vagus nerve (Molina-Holgado *et al.*, 2005, Van *et al.*, 2005) as well as on cerebromicrovascular endothelial cells (Golech *et al.*, 2004) and microglia (Carlisle *et al.*, 2002). Its predominant location in cells of the immune system has led researchers to believe that it is mainly involved in the immunomodulatory actions of cannabinoids.

Both the CB_1R and CB_2R are class I G-protein-coupled receptors (GPCRs) and have the classic 7 transmembrane (TM) domain structure. The basic structures of the human CB_1R and CB_2R are shown in Fig. 1.6. The CB_1R couples to Gi/o-proteins and it has been shown that multiple conformations of the receptor can be induced by ligands in order to regulate individual G-proteins (Glass & Northup, 1999, Prather *et al.*, 2000, Mukhopadhyay & Howlett, 2005) and that the CB_1R can sequester Gi/o-proteins, making them unavailable to other GPCRs (Vasquez & Lewis, 1999). CB_1R coupling to Gs (Glass & Felder, 1997, Bonhaus *et al.*, 1998, Jarrahian *et al.*, 2004) and Gq-proteins (Lauckner *et al.*, 2005) has also been noted. The CB_2R couples to Gi-proteins but interacts less efficiently with Go-proteins (Glass & Northup, 1999). It has been demonstrated that both receptors are constitutively active (Bouaboula *et al.*, 1997, Bouaboula *et al.*, 1999, Portier *et al.*, 1999) and they are capable of linking to a number of different signalling pathways, some of which will be discussed in further detail later, such as inhibition of cAMP production and activation of MAPKs such as extracellular signal-regulated kinases-1 and –2 (ERK1/2; Demuth & Molleman, 2006).

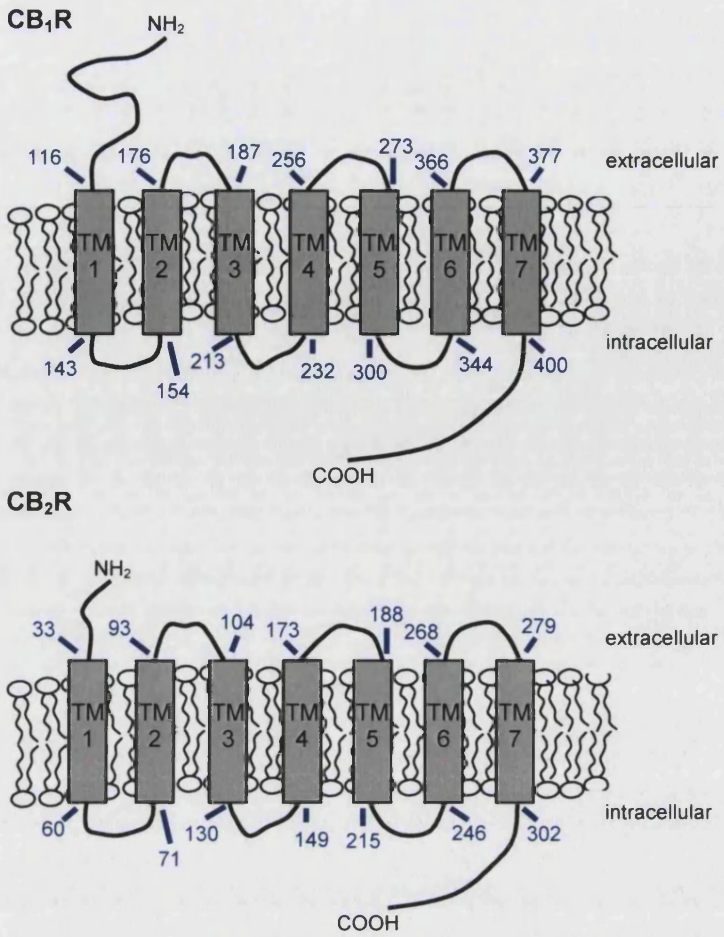


Figure 1.6: **Basic structure of the human CB_1R and CB_2R.** This cartoon symbolises the 7 TM topology of the CB_1R and CB_2R. The amino acid numbers that mark the beginning and end of each of the proposed TM domains are shown. Details of the amino acid sequence of both receptors can be found in *Appendices*.

It is believed that cannabinoid agonists bind the CB_1R within the pore formed within the TM helical cluster (Mukhopadhyay *et al.*, 2002, Murphy & Kendall, 2003) although certain portions of the extracellular loops may also play a role in ligand binding (Murphy & Kendall, 2003). AEA, for instance, is believed diffuse through the lipid bilayer and then interact with a groove in the sixth TM domain (Makriyannis *et al.*, 2005). Similarly the TM domains of the CB_2R have been shown to be important in ligand binding and specificity (Shire *et al.*, 1999, Xie *et al.*, 2003). The three cytosolic loops and the intracellular C-terminal domain of the CB_1R all contribute to the activation of G-proteins (Mukhopadhyay *et al.*, 2002). For example, the juxtamembrane C-terminal domain of the human CB_1R is critical for G-protein coupling whilst the distal C-terminal domain is important for modulating the magnitude and kinetics of signal transduction (Nie & Lewis, 2001). In the CB_2R the areas involved in G-protein

coupling may be different as the juxtamembrane C-terminal domain of CB_2R appears not to be involved in G-protein coupling (Mukhopadhyay & Howlett, 2001). The CB_1R also has an exceptionally long N-terminal domain which has been found to be involved in receptor stability and limits its expression at the plasma membrane (Andersson *et al.*, 2003). It has also been recently discovered that the CB_1R contains an allosteric binding site that can be recognised by a class of small synthetic ligands Org 27569, Org 27759 and Org 29647 although the implications of this are not yet known (Price *et al.*, 2005).

Many GPCRs are known to form oligomers such as homodimers and heterodimers. It is a field that has gathered much interest as it has been shown that heterodimerisation can give rise to receptor complexes that bind ligands, signal and traffic differently from the component receptors (Maggio *et al.*, 2005). There is evidence to suggest that the CB_1R can exist as homodimers (Wager-Miller *et al.*, 2002, Mackie, 2005, Xu *et al.*, 2005) as well as form heterodimers with GPCRs such as the D2 receptor (Kearn *et al.*, 2005), lysophosphatidic acid (LPA) 1 receptor (LPA1, Edg2; Wright *et al.*, 2005), orexin 1 receptor (Hilairet *et al.*, 2003) and possibly opioid receptors (Rios *et al.*, 2006). CB_2R homodimers have also been suggested to exist (Filppula *et al.*, 2004)

Exposure of GPCRs to agonists often results in rapid attenuation of receptor responsiveness, a process referred to as receptor desensitisation. This involves uncoupling of the receptor from its G-proteins in response to receptor phosphorylation, internalisation of cell surface receptors to intracellular compartments, and downregulation of the total receptor numbers (Fig. 1.7). However, the term desensitisation is often used just to refer to the process of G-protein uncoupling. The term homologous desensitisation denotes agonist-specific desensitisation whereby only the active receptor is regulated. This in contrast to heterologous desensitisation which is not agonist-specific and inactive receptors are regulated by other receptors coupling to the same or different signalling pathways. The extent of receptor desensitisation can vary between systems from complete termination of signalling to attenuation of agonist potency and maximal responsiveness (Ferguson, 2001, Kristiansen, 2004).

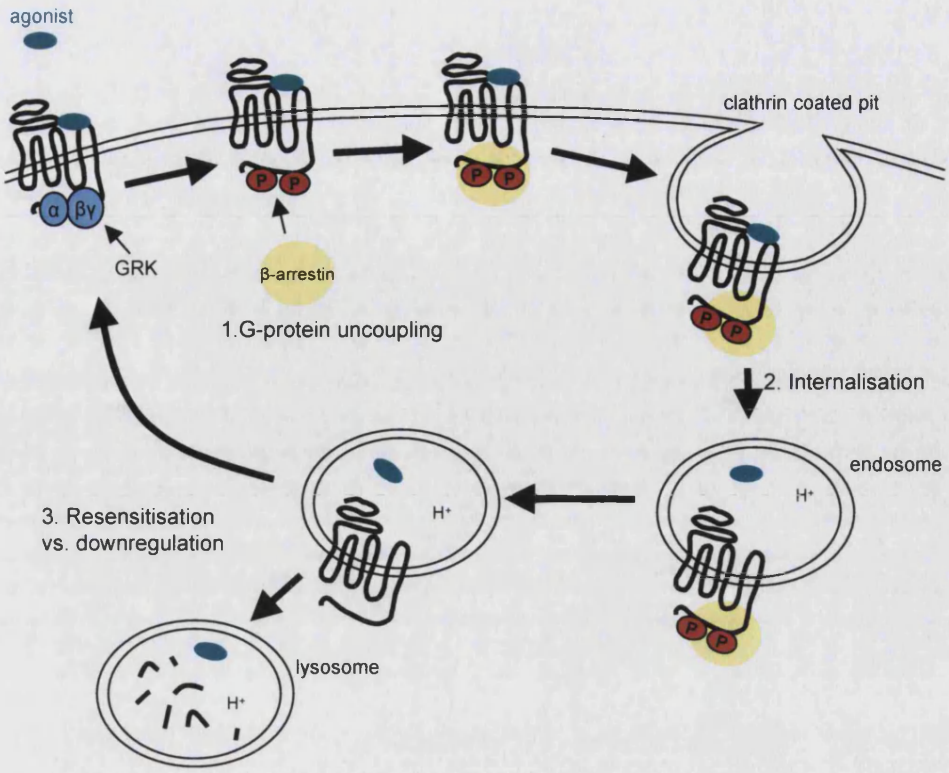


Figure 1.7: **Basic outline of homologous GPCR desensitisation and resensitisation.** The waning of GPCR responsiveness to agonist with time is important in protecting against receptor overstimulation and this desensitisation involves several mechanisms. 1) Heterotrimeric G-protein uncoupling: G-protein uncoupling occurs in response to phosphorylation of the intracellular portions of the receptor (predominantly the third intracellular loop and C-terminal domain) by 2nd messenger-dependent protein kinases such as cAMP-dependent protein kinase (PKA) and PKC, and G-protein-coupled receptor kinases (GRKs). GRKs selectively phosphorylate agonist-activated receptors and increase the affinity of β-arrestin binding. β-arrestins physically uncouple the G-protein from the receptor and also target GPCRs for internalisation via clathrin-coated vesicles. 2) Receptor internalisation: the rate of internalisation is receptor specific and it has been suggested that multiple endocytic mechanisms exist. Many GPCRs internalise in a β-arrestin and clathrin-dependent manner as depicted, but calveolae, which can contain proteins involved in vesicle formation, represent an alternative route for some. β-arrestins target GPCRs to clathrin-coated pits by associating with a number of proteins involved in endocytosis such as the AP-2 adaptor complex which is involved in promoting clathrin coat assembly and clathrin itself. 3) Receptor recycling versus downregulation: some receptors are recycled back to the cell surface, once β-arrestin is removed and they are dephosphorylated, thereby resensitising the cell to the agonist. However, other GPCRs are internalised and then retained in endosomes and/or targeted to lysosomes for degradation. The pathway followed by specific GPCRs is regulated by determinants in the C-terminus. Degradation of these internalised receptors, alongside a decrease in receptor mRNA and protein sysnthesis, constitutes receptor downregulation (Ferguson, 2001, Kristiansen, 2004).

Following cannabinoid exposure the CB_1R has been demonstrated to desensitise in a GRK and β-arrestin-dependent manner (Jin *et al.*, 1999). It has also been shown to internalise rapidly (Jin *et al.*, 1999, Hsieh *et al.*, 1999), via clathrin-coated pits (Hsieh *et al.*, 1999) and caveolae (Keren & Sarne, 2003), and agonists vary in their potency with regard to stimulating internalisation (Hsieh *et al.*,

1999). Following short-term cannabinoid treatment the CB_1R can be recycled back to the cell surface (Hsieh *et al.*, 1999). Different sections of the C-terminal domain are thought to be involved in desensitisation and internalisation (Hsieh *et al.*, 1999, Jin *et al.*, 1999). The C-terminus is also thought to play an important role in CB_2R desensitisation and internalisation (Bouaboula *et al.*, 1999). Chronic administration of cannabinoids *in vivo* induces downregulation of the CB_1R in several brain areas (Martin *et al.*, 2004), suggesting that whereas short term agonist exposure appears to cause reversible internalisation of the CB_1R, long term treatment leads to internalisation and receptor downregulation (Hsieh *et al.*, 1999).

Cannabinoid receptor expression is not only modulated by cannabinoid exposure but also by cellular activation and differentiation, as seen in immune cells. For example, it was found that CB_1R mRNA expression increased when PBMCs were cultured for up to 24 hours in media and that this was counteracted if the cells were stimulated with the T lymphocyte mitogen phytohemagglutinin (PHA; Nong *et al.*, 2002). In addition, it has been shown that levels of both CB_2R mRNA and protein are modulated during B cell differentiation from virgin B cells through to memory B cells (Carayon *et al.*, 1998, Marchand *et al.*, 1999).

1.1.3.2 Additional cannabinoid-like receptors

In addition to the CB_1R and CB_2R, other cannabinoid-like receptors have been suggested to exist (Howlett *et al.*, 2002, Wiley & Martin, 2002, Begg *et al.*, 2005, Demuth & Molleman, 2006). For example, an endothelial cannabinoid receptor has been proposed to exist based on the findings that AEA can cause vasodilatation in isolated mesenteric arterial beds that is dependent on the presence of endothelial cells, cannot be reproduced by synthetic non-selective cannabinoid agonists and is only inhibited by concentrations of the CB_1R antagonist, SR141716A, above those needed to block the CB_1R. Abnormal cannabidiol (abn-cannabidiol), a non-psychotropic cannabinoid, also stimulates this receptor and the response was found to be VR1-independent (Begg *et al.*, 2005).

Although non-classical, cannabinoid-like, receptors have been reported, a phylogenetic analysis of the cannabinoid receptors has revealed no additional cannabinoid receptor homologue (Anday & Mercier, 2005). One possibility is that some of the responses detected that do not conform to a typical CB_1R or CB_2R-mediated response are due to the formation of oligomers, either homo or heterodimers, between the cannabinoid receptors or with other GPCRs (Mackie, 2005). It is well documented that dimerisation can alter the pharmacology of GPCRs (Maggio *et al.*, 2005) and CB_1R homodimerisation as well as association with other GPCRs has already been reported as mentioned above (Kearn *et al.*, 2005, Mackie, 2005, Wright *et al.*, 2005). Another possibility is that these responses are mediated by cannabinoid receptor splice variants. Thus far two splice variants of the CB_1R with unique pharmacological profiles have been described (Ryberg *et al.*, 2005). There are also a number of additional receptors that the cannabinoids can couple to, as discussed below, and these may be involved in some of the effects seen, as may the endocannabinoid metabolites.

Furthermore, it is also possible that additional cannabinoid receptor subtypes do exist but that they exhibit little identity to the CB_1R and CB_2R. One recent paper has reported that two separate patents indicate that the orphan GPCR, GPR55, is a novel cannabinoid receptor (Baker *et al.*, 2006). Several cannabinoids, including CP55,940, AEA, 2-AG and AM251, were shown to bind GPR55, whilst others such as WIN55,212-2 did not. The receptor exhibits low identity (10-15%) to both the CB_1R and CB_2R but there are certain conserved sequences between the putative TM domains of GPR55 and the CB_1R and CB_2R. In contrast to the CB_1R and CB_2R, GPR55 does not couple to Gi or Gs-proteins. It appears to be expressed in a number of human tissues, including certain brain regions and the spleen.

1.1.3.3 VR1

The VR1 (also known as TRPV1) is a non-selective cation channel that is expressed in sensory neurons in the peripheral nervous system and in various brain regions. It is a member of transient receptor potential channel superfamily. It is involved in nociception and responds to a number of different stimuli

including several chemical stimuli such as capsaicin, AEA and other NAEs, NADA, resiniferatoxin, gingerols, and several lipoxygenase products (Smart *et al.*, 2000, Caterina & Julius, 2001, Dedov *et al.*, 2002, Huang *et al.*, 2002, Hwang & Oh, 2002, Ross, 2003, Movahed *et al.*, 2005). The lipid mediators all bind intracellularly. Activity at the VR1 by these ligands is reported to be regulated by several additional factors including phosphorylation of the VR1 by PKC (Ross, 2003) and acidic conditions (Caterina & Julius, 2001). VR1-dependent actions of AEA include induction of apoptosis in tumour cell lines (Maccarrone *et al.*, 2000) and vasodilatation in isolated rat arteries (Zygmunt *et al.*, 1999).

1.1.3.4 Additional receptors cannabinoids may interact with

Cannabinoids have also been shown to interact with several receptors other than the cannabinoid receptors and the VR1 and it is likely that this list will continue to grow in the future. For instance, AEA can inhibit 5-HT-induced currents through the human 5-HT_{3A} receptor in a cannabinoid receptor-independent manner (Barann *et al.*, 2002). It was suggested that AEA acts allosterically at a modulatory site on the 5-HT_{3A} receptor. Cannabidiol, one of the natural plant cannabinoids has also been shown to act as an agonist at the human 5-HT_{1A} receptor and it has been suggested that it is acting at the ligand-binding site of the receptor (Russo *et al.*, 2005). Furthermore, it has been reported that AEA and 2-AG directly affect the functioning of inhibitory glycine receptor channels (Lozovaya *et al.*, 2005) and that AEA can inhibit agonist binding to M_1 muscarinic acetylcholine receptors (Lanzafame *et al.*, 2004). Several cannabinoids have been shown to activate PPAR-γ, including AEA and 2-AG as previously mentioned (Burstein, 2005). For example, ajulemic acid was found to bind to and activate the transcriptional activity of PPAR-γ (Liu *et al.*, 2003) and Δ^9-THC was also shown to activate PPAR-γ (O'Sullivan *et al.*, 2005). Furthermore, 2-AG, ajulemic acid and Δ^9-THC were all shown to stimulate adipocyte differentiation in cultured 3T3L1 cells, a well accepted property of PPAR-γ ligands (Liu *et al.*, 2003, Burstein, 2005, O'Sullivan *et al.*, 2005). In addition, PEA, another NAE, has recently been shown to activate PPAR-α (Lo Verme *et al.*, 2005a).

1.1.4 Cannabinoids and cellular signal transduction

The cannabinoids have been linked to numerous downstream signalling pathways including ion channel activation, MAPK activation and regulation of cAMP production via the cannabinoid receptors. This is not meant to be an exhaustive review on cannabinoid signalling but rather an introduction to some of the downstream effectors that have been more widely studied. As well as cannabinoid receptor-mediated effects, there are also suggestions of receptor-independent effects. However, much of this data is largely based on the use of CB_1R and CB_2R antagonists such as SR141716A and SR144528. In light of the possible inverse agonism of these compounds (Bouaboula *et al.*, 1999) and the suggested existence of additional cannabinoid receptors (Wiley & Martin, 2002, Begg *et al.*, 2005) further work may be required to verify these claims.

The lipid bilayer appears to play an important role in cannabinoid signalling. Cholesterol enrichment of membranes has been shown to decrease CB_1R binding and also decrease subsequent downstream signalling, whereas cholesterol depletion enhances binding and downstream signalling events, although actual levels of CB_1R expression appeared to be unaffected (Bari *et al.*, 2005a, Bari *et al.*, 2005b). Other studies have shown that membrane cholesterol depletion can inhibit AEA or met-AEA-induced signalling that is independent of the cannabinoid receptors and VR1 (Sarker & Maruyama, 2003, Hinz *et al.*, 2004). Thus, a novel role for lipid rafts, which are highly dynamic and discreet membrane domains rich in sphingolipids and cholesterol (Rajendran & Simons, 2005), has been suggested in cannabinoid signalling. It may be that the lipid rafts play a role in determining whether receptor-dependent or independent events occur, alter AEA cellular accumulation or that they represent an additional mechanism of signal transduction.

1.1.4.1 MAPK activation

The MAPK family members, including ERK1/2 (p42/p44), p38 and c-Jun N-terminal kinase (JNK), play key roles in several cellular functions such as cell

proliferation and differentiation. In resting cells the MAPKs largely reside in the cytoplasm but can enter the nucleus once activated to phosphorylate nuclear substrates such as transcription factors, protein kinases and cell cycle regulators. Each MAPK is activated through a cascade of successively activating protein kinases: MAPK kinase and MAPK kinase kinase. The basic outline of these pathways is shown in Fig. 1.8. There are multiple family members for each kinase, in particular there are many MAPK kinase kinases, and each is differentially regulated in part through interactions with G-proteins, scaffolds, chaperones, adaptors, kinases, phosphatases, substrates, regulatory proteins and lipids.

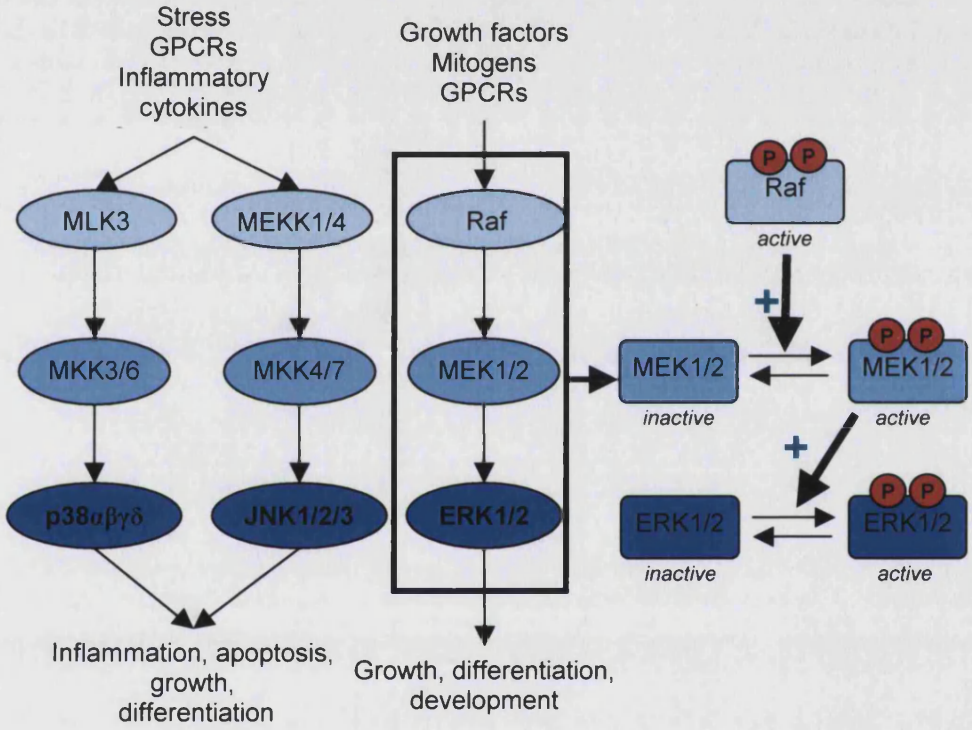


Figure 1.8: **Schematic representation of the structure of MAPK pathways.** Briefly, the pathway consists of a cascade of three kinases: a MAPK kinase kinase (•) that phosphorylates and activates a MAPK kinase (•) which then activates the MAPK (•). The ERK1/2 activation pathway is shown in more detail as an example. Full activation of the Raf serine/threonine kinases (Raf-1, A-Raf and B-Raf) requires several steps, including several phosphorylations by upstream kinases. Once activated, Raf phosphorylates MAPK/ERK kinase-1/2 (MEK1/2) on serine residues and, in turn, this dual specificity kinase then activates ERK1/2 via phosphorylation of a Thr-Glu-Tyr motif in the activation loop. This phosphorylation can be detected, for instance by using antibodies that bind the phosphorylated form of the protein, and used as a marker of activation. Once activated, ERK1/2 translocates to the nucleus and there regulates the activity of several transcription factors such as Elk-1 (Kolch, 2000, Pouyssegur *et al.*, 2002, Anderson, 2006). *Abbreviations:* MEKK, MEK kinase; MKK, mitogen-activated protein kinase kinase; MLK, mixed lineage kinase.

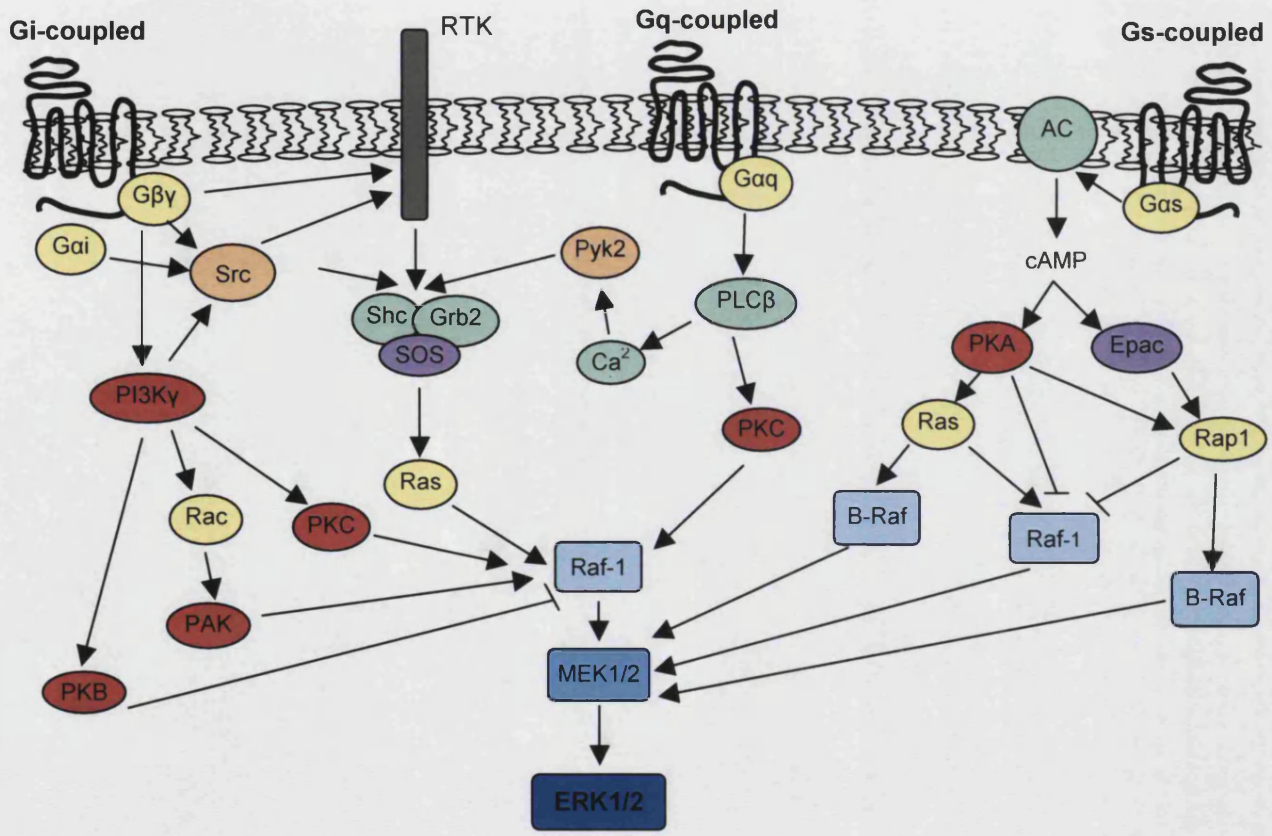
Several studies have shown that cannabinoids can couple to these MAPKs and thereby have effects on a number of cellular activities or further signalling pathways. For example, MAPK has been shown to be involved in cannabinoid-induced glucose metabolism (Sanchez *et al.*, 1998), activation of the Na^+/H^+ exchanger NHE-1, which is involved in control of cell volume in CHO-CB_1R cells (Howlett & Mukhopadhyay, 2000) and inducing expression of immediate early genes such as krox-24 (Bouaboula *et al.*, 1995a).

Wartmann *et al* (1995) were the first to report ERK activation by an endocannabinoid, namely AEA, and Bouaboula *et al* (1995b) were the first to demonstrate that the CB_1R can couple positively to ERK1/2 phosphorylation and activation via a pertussis toxin (PTX)-sensitive and cAMP-independent mechanism. Since then a number of *in vitro* studies have been done to show that ERK can be activated by cannabinoids in a CB_1R and CB_2R-dependent manner (Bouaboula *et al.*, 1996, Liu *et al.*, 2000, Kobayashi *et al.*, 2001, Galve-Roperh *et al.*, 2002, Gomez *et al.*, 2002, Samson *et al.*, 2003, Herrera *et al.*, 2005). In addition, the *in vivo* administration of Δ^9-THC has been linked to the induction of ERK activation in murine dorsal striatum and nucleus accumbens (Valjent *et al.*, 2001), hippocampus (Derkinderen *et al.*, 2003) and rat cerebellum (Rubino *et al.*, 2004). Cannabinoids have also been shown to couple negatively to ERK activation. For example, AEA has been reported to inhibit sustained ERK activation in PC12 cells induced by nerve growth factor (Rueda *et al.*, 2002) and cannabinoids appear to inhibit phorbyl 13-myristate 12-acetate (PMA)/Ionomycin-induced ERK activation in mouse splenocytes (Faubert & Kaminski, 2000, Faubert Kaplan & Kaminski, 2003).

As shown in Fig. 1.8 the basic pathway leading to ERK1/2 activation involves the kinases Raf and MEK1/2 (Kolch, 2000, Pouyssegur *et al.*, 2002, Anderson, 2006). GPCRs can couple to this pathway in many different ways and the exact pathway involved in ERK1/2 activation varies between receptor and cell types but some of the major pathways are outlined in Fig. 1.9. For example, activation of Gq-protein coupled receptors leads to ERK1/2 activation in either a PKC and/or Ras-dependent manner. PKC can both directly and indirectly enhance the activity of Raf-1 and Ras is a GTPase that binds Raf when active, thereby

localising it to the plasma membrane, one of the steps in Raf activation. Activation of, for instance, Gi-protein-coupled receptors, and release of the Gβγ-subunits, can also lead to ERK1/2 activation via many different routes, including receptor transactivation. Transactivation occurs when activation of a GPCR leads to stimulation of a receptor tyrosine kinase (RTK), such as epidermal growth factor receptor (Marinissen & Gutkind, 2001, Werry *et al.*, 2005). This creates docking sites for proteins that contain phosphotyrosine binding domains and leads to the assembly of complexes containing son of sevenless (SOS) which will in turn activate Ras. Activation of Gs-protein coupled receptors can lead to either inhibition or stimulation of ERK1/2 activation via the generation of cAMP (Stork & Schmitt, 2002, Bos, 2003, Stork, 2003). In addition, there may also be a role for endocytosis, and proteins such as dynamin and arrestin which are involved in this process, in ERK1/2 activation downstream of some, but not all, GPCRs (Werry *et al.*, 2005).

Current information on how the cannabinoid receptors couple to ERK1/2 activation indicates that the pathways may be cell specific, however, all the reports agree that a PTX-sensitive G-protein is involved. One of the ways in which the cannabinoid receptor could link to ERK activation is through the inhibition of cAMP as the possible links between cAMP and ERK1/2 are numerous (Fig. 1.9; Stork & Schmitt, 2002, Bos, 2003, Stork, 2003). Whilst there are some studies that show that cannabinoid-induced ERK1/2 activation is a cAMP-independent process (Bouaboula *et al.*, 1995b, Bouaboula *et al.*, 1996, Sanchez *et al.*, 1998) other suggest that a decrease in cAMP levels, and subsequently PKA activity, may play a role in the stimulatory effects of cannabinoid receptor activation on ERK activation (Melck *et al.*, 1999, Davis *et al.*, 2003, Derkinderen *et al.*, 2003). A link between the release of ceramide through sphingomyelin hydrolysis induced by cannabinoids acting through the CB_1R and consequent activation of the Raf-1/ERK1/2 pathway has also been suggested (Sanchez *et al.*, 1998). In accordance with a role for Raf in ERK activation there have been several reports indicating that cannabinoids can enhance Raf-1 translocation and activity (Sanchez *et al.*, 1998, Melck *et al.*, 1999, Galve-Roperh *et al.*, 2000, Galve-Roperh *et al.*, 2002, Sanchez *et al.*, 2003).



Abbreviations:
AC, adenylyl cyclase;
Epac, exchange factor directly activated by cAMP;
Grb2, growth factor receptor-bound 2;
PAK, p21 activated kinase;
PI3K, phosphoinositide 3-kinase;
PKB, protein kinase B;
PLC, phospholipase C;
Pyk2, proline-rich tyrosine kinase-2;
Shc, Src homology and collagen.

Figure 1.9: **Schematic outlining some of the major pathways linking GPCRs to ERK1/2 activation.** Notably many of the connections shown are not direct and involve additional scaffolding proteins and/or modulators. However, some of the key regulators of ERK1/2 activation are shown and details of these pathways can be found in the following reviews: Kolch, 2000; Marinissen & Gutkind, 2001; Bos, 2003; Stork, 2003; Werry *et al*, 2006.

Furthermore, both the CB_1R and CB_2R have also been shown to couple to ERK1/2 via PKC (Bouaboula *et al.*, 1996, Liu *et al.*, 2000). A role for PI3K in cannabinoid-induced ERK activation has also been demonstrated in several different studies using the PI3K inhibitors LY294002 and wortmannin (Bouaboula *et al.*, 1997, Galve-Roperh *et al.*, 2002, Samson *et al.*, 2003, Sanchez *et al.*, 2003). It has also been suggested that the non-receptor tyrosine kinase, Src, may be required for maximal ERK activation as two studies have shown that Src inhibition decreased cannabinoid-induced ERK activation although the cannabinoids did not stimulate Src *per se* (Galve-Roperh *et al.*, 2002, Davis *et al.*, 2003). Similarly, another Src-family kinase, Fyn, was proposed to have a role in cannabinoid-induced ERK activation in two further studies (Derkinderen *et al.*, 2003, Zhao *et al.*, 2005). Some of the pathways currently thought to link the cannabinoid receptors and ERK1/2 activation are briefly outlined in Fig. 1.10.

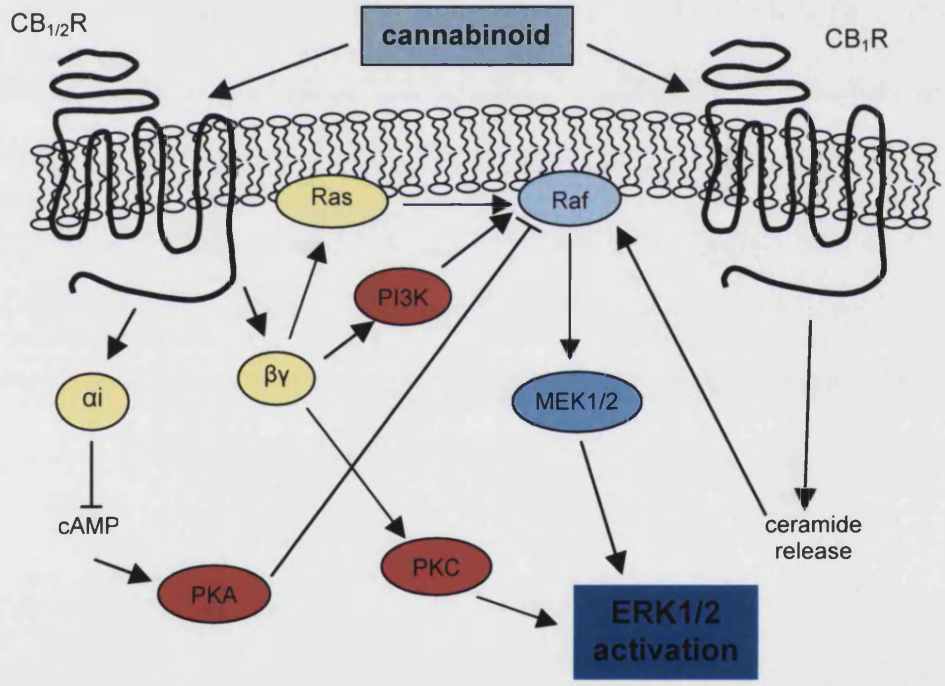
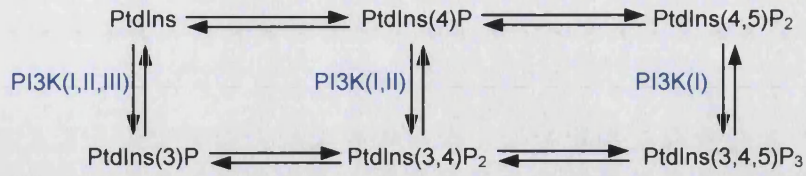


Figure 1.10: **A schematic of some of the pathways currently thought to link the cannabinoid receptors to ERK1/2 activation.** Many of the intermediary steps in these pathways have not yet been examined in the context of cannabinoid signalling and have therefore not been represented here. In addition, one study has shown that ERK activation occurred downstream of epidermal growth factor receptor transactivation in number of different cell lines, however, they did not investigate the role of the cannabinoid receptors in this response and it has therefore not been included on this diagram (Hart *et al.*, 2004).

Some studies have also reported receptor-independent activation of ERK by AEA. For instance, AEA-induced ERK activation in CB_2R transfected CHO cells was only reduced by about 50% by the CB_2R-selective antagonist SR144528 and also occurred in wild type CHO cells (Derocq *et al.*, 1998). The authors also showed that AA also stimulated ERK activation to a similar degree as AEA but did not further explore the receptor-independent mechanism of action. Similarly, AEA-induced ERK activation in ECV304 human umbilical vein endothelial cells was only partially inhibited by the CB_1R-selective antagonist SR141716A or transfection with CB_1R antisense oligonucleotides suggesting the involvement of additional pathways although again these were not further explored (Liu *et al.*, 2000).

In addition to ERK1/2, cannabinoids have also been shown to activate JNK and p38. Both JNK1 and JNK2 were stimulated by cannabinoids in CB_1R transfected CHOs, with data supporting the involvement of a Gi/o-protein, PI3K and Ras (Rueda *et al.*, 2000). RTK transactivation was also though to play a role but not ceramide production. CB_1R-dependent activation of JNK1 and JNK2 was also detected in rat cortical neurons (Downer *et al.*, 2003). Both JNK and p38 activation downstream of cannabinoids was also shown in several cell types such as ECV304 cells (Liu *et al.*, 2000) and Jurkat human leukaemia cells (Herrera *et al.*, 2005). In addition, cannabinoid receptor-dependent stimulation of p38 has been demonstrated in CB_1R transfected CHO cells (Rueda *et al.*, 2000) and rat and murine hippocampal slices (Derkinderen *et al.*, 2001a). In contrast, neither JNK, p38 nor ERK1/2 was activated by cannabinoids in rat cortical neurons (Molina-Holgado *et al.*, 2005) underlining the fact that although cannabinoids can couple to the MAPKs the responses are cell-type specific.

1.1.4.2 Activation of PI3K/PKB-dependent signalling



Class	Subunits		Tissue distribution	Regulation
	catalytic	adaptor		
IA	p110α,β,δ	p85α,β p55α,β p50α	α,β - ubiquitous δ - leukocytes	tyrosine kinases, Ras, Gβγ (p110β)
IB	p110γ	p101 p84	leukocytes	Gβγ, Ras
II	C2α,β,γ	clathrin Grb2	α,β - ubiquitous γ - hepatocytes	tyrosine kinases, chemokines, integrins
III	Vps34p	p150	ubiquitous	constitutively active *in vitro*

Figure 1.11: ***In vitro* lipid substrate specificity of the PI3K enzymes and their basic subunit components, tissue distribution and regulation.** The PI3K family is divided into three classes based on their *in vitro* lipid substrate specificity, structure and likely modes of regulation. Class I, which is further subdivided into two types, A and B, is the best characterised to date. Each class I PI3K is a heterodimer consisting of a catalytic subunit and an adaptor subunit. An interaction of the adaptor proteins with phosphotyrosines links the Class IA PI3K isoforms with tyrosine kinase signalling pathways whilst the Class IB PI3K, p110γ, couples to GPCRs signaling pathways through its adaptor proteins. In addition, p110β has also been shown to link to GPCR signalling and all the Class I isoforms can be regulated by Ras (Maier *et al.*, 1999, Brock *et al.*, 2003, Foster *et al.*, 2003, Suire *et al.*, 2005). Relatively little is known about the Class II and III PI3Ks although it is emerging that Class II PI3Ks may be activated downstream of several extracellular signals including integrin engagement, chemokines and growth factors, and that class III may be constitutively active and involved in intracellular trafficking events such as vesicular transport (Foster *et al.*, 2003). *Abbreviations:* PtdIns, phosphatidylinositol.

The PI3Ks are a family of kinases that phosphorylate PI lipids at the D-3 position to create a variety of lipid mediators and thereby regulate a multitude of biological functions (Foster *et al.*, 2003). Their *in vitro* lipid substrate specificity, subunit composition, tissue distribution and regulation are outlined in Fig. 1.11. One of the downstream effectors of PI3K is PKB (also referred to as Akt), a serine/threonine protein kinase which is involved in many cellular functions including regulation of protein synthesis and cell survival pathways. There are three isoforms, α, β and γ and their activation involves the phosphorylation of two residues, Ser473 and Thr308 (in PKBα; Vanhaesebroeck

& Alessi, 2000, Song *et al.*, 2005, Woodgett, 2005). The exact mechanism of activation has not yet been resolved but the events thought to occur are depicted in Fig. 1.12.

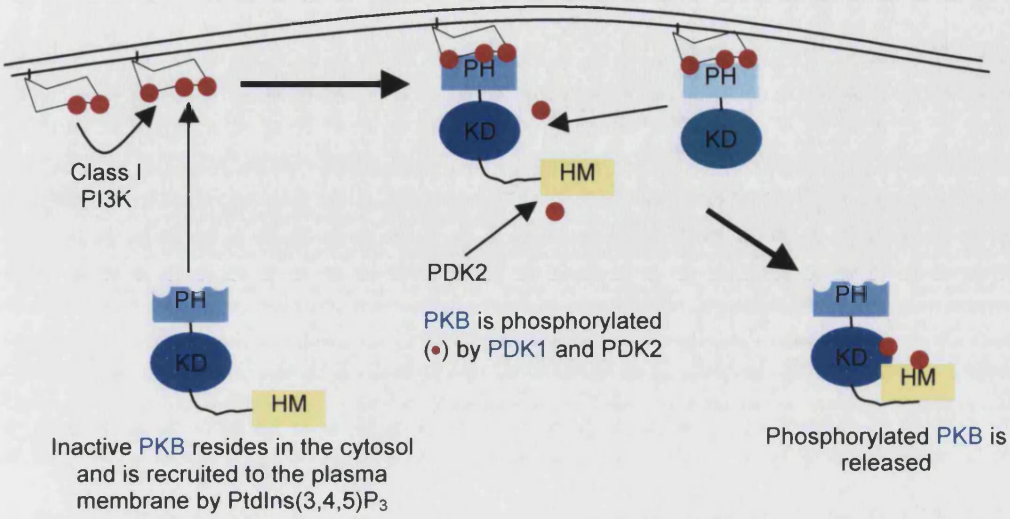


Figure 1.12: **The mechanism of activation of PKB.** The production of PtdIns(3,4,5)P_3 through the activation of class I PI3K is the first step in PKB activation by most stimuli. This lipid mediator recruits PKB to the plasma membrane through interaction with its N-terminal pleckstrin homology (PH) domain. It is thought that this interaction induces a conformation of PKB that is more accessible to 3'phosphoinositide-dependent kinase-1 (PDK1) which phosphorylates PKB on Thr308 (of PKBα) in its kinase domain (KD). Constitutively active, PDK1 colocalises with PKB through interaction between its PH domain and PIs. An as of yet unidentified kinase, generally referred to as PDK2, phosphorylates PKB on Ser473 (of PKBα) in the C-terminal hydrophobic motif. This phosphorylation may be important in PDK1-mediated phosphorylation of Thr308. Candidates for PDK2 include PKB itself, integrin-linked kinase, DNA-dependent protein kinase, PKCβII and a rictor-mammalian target of rapamycin complex. Both residues must be phosphorylated for full PKB activation. Once active, PKB dissociates from the membrane and translocates to its sites of action (Vanhaesebroeck & Alessi, 2000, Sarbassov *et al.*, 2005, Song *et al.*, 2005, Woodgett, 2005). PKB function is in part controlled by dephosphorylation of PtdIns(3,4,5)P_3, for instance by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) as well as a dephosphorylation of Ser473 by PH domain leucine-rich repeat protein phosphatase (Gao *et al.*, 2005). Other PKB binding proteins also exist and could further modulate PKB activity (Anai *et al.*, 2005, Song *et al.*, 2005).

Cannabinoid receptors appear to couple to PI3K (Bouaboula *et al.*, 1997, Galve-Roperh *et al.*, 2002) and thus it follows that PKB could be activated downstream of these receptors. Indeed, transient activation of PKB via the CB_1R has been shown in several different cell types including U373 MG human astrocytoma cells and rat primary cortical neurons (Gomez *et al.*, 2000, Galve-Roperh *et al.*, 2002, Molina-Holgado *et al.*, 2002, Molina-Holgado *et al.*, 2005) and is thought to be involved in the neuroprotective actions of cannabinoids. Initially it was

suggested that the CB_2R may not couple to PKB activation as cannabinoid-induced PKB activation was not detected in HL-60 cells which express the CB_2R (Gomez *et al.*, 2000). However, subsequent data from rat primary cortical neurons demonstrated that HU210 elicited PKB phosphorylation, a critical step in activation. This response was downregulated by AM630, a CB_2R-selective antagonist (Molina-Holgado *et al.*, 2005). Furthermore, cannabinoid-induced PKB phosphorylation in PC-3 human prostate epithelial cells could be inhibited by SR144528, another CB_2R-selective antagonist (Sanchez *et al.*, 2003). In fact, in mast cells it was shown that the CB_2R, but not the CB_1R, couples to PKB phosphorylation (Samson *et al.*, 2003). This suggests that the CB_2R may couple to PKB in some cell types. There has also been one recent study showing that in C6 glioma cells the non-selective agonist WIN55,212-2 downregulated PKB signalling over 48 hours (Ellert-Miklaszewska *et al.*, 2005).

1.1.4.3 Activation of PKC

The PKC family of serine/threonine protein kinases is composes of at least 10 isoforms which have distinct differences in structure, expression and activation requirements (Fig. 1.13). PKC is activated downstream of many different stimuli including those that activate GPCRs and are involved in a number of cellular functions such as cell migration and T lymphocyte activation and have a number of substrates including Raf. They are generally widely distributed in mammalian tissues although some isoforms are more restricted. For example, PKCγ is mainly found in the CNS and spinal cord, PKCθ in skeletal muscle and haematopoietic cells and PKCβ in pancreatic islet cells, monocytes, brain and retinal tissue. PKCs can interact with a myriad of different proteins some of which, such as receptors for activated PKCs (RACKs), target activate PKCs to specific membrane compartments and thereby play a role in determining isoform-specific functions (Way *et al.*, 2000, Tan & Parker, 2003, Parker & Murray-Rust, 2004, Larsson, 2006).

Group	Isoforms	Requirements for activation
cPKC	α, βI, βII, γ	•Ca^{2+}-dependent •activated by PS & DAG
nPKC	δ, ε, η, θ	•Ca^{2+}-insensitive •activated by DAG in the presence of PS
aPKC	ζ, ι/λ	•Ca^{2+}-insensitive •do not respond to DAG •activity regulated by PS

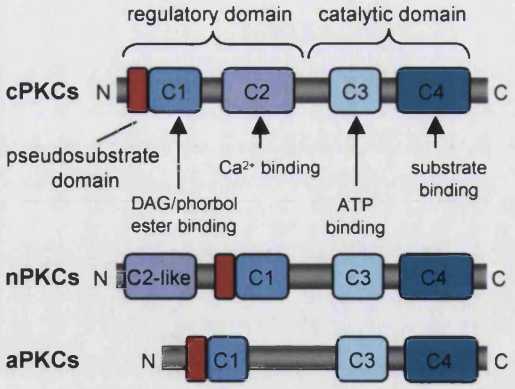


Figure 1.13: **The different PKC isoforms and how they are activated.** Several PKC isoforms exist and they are divided into three groups based on their structure and requirements for activation. Inactive PKC resides in the cytosol and activation is multistep process which involves removing an inhibitory pseudosubstrate domain from the active site such that the enzyme can bind and phosphorylate its substrates. Conventional (c)PKCs are activated by the phospholipid phosphatidylserine (PS) in a Ca^{2+}-dependent manner. In addition they bind DAG which not only increases their affinity for PS but also shifts the requirement for Ca^{2+} into the physiological range. cPKCs can also be activated by phorbol esters such as PMA. Conserved (C) domains, C1 and C2 in the regulatory region of the protein, are required for interaction with DAG/phorbol esters and PS and Ca^{2+}, respectively. Although the novel (n)PKCs and atypical (a)PKCs have a C2-like domain, this region does not bind Ca^{2+} and thus these enzymes are Ca^{2+}-insensitive. However, nPKCs are activated by DAG/phorbol esters in the presence of PS unlike the aPKCs. The C4 domain of PKC is the substrate binding site and lies within the C-terminal catalytic region. Conformational changes associated with PS binding displaces the pseudosubstrate domain out of the active site in the C4 region but for optimum catalytic activity all PKC isoforms must also be phosphorylated in their activation loop by PDK1 (Thr500 in PKCβII). Additional phosphorylation sites also exist for the cPKCs and nPKCs and these all act in concert to lock the kinase in its active form (Way *et al.*, 2000, Parker & Murray-Rust, 2004).

PKC was shown to be involved in AEA-mediated ERK activation in ECV304 cells and CP55,940-induced ERK activation in CB_2R transfected CHO cells using the PKC-specific inhibitor GF 109203X (Bouaboula *et al.*, 1996, Liu *et al.*, 2000). However, few studies have investigated cannabinoid-mediated effects on PKC directly. In one *in vitro* system the effects of PS, dioleylglycerol and AEA on Ca^{2+}-induced rat brain PKC activity were examined. The authors reported a dual modulation of PKC activity. In the presence of AEA, the PS-induced activity of PKC was increased but DAG-mediated activation was inhibited (De Petrocellis *et al.*, 1995). They suggested that AEA binds to the DAG site on PKC and therefore has a direct effect on PKC activity rather than a receptor-mediated one. However, in another study using human keratinocytes, AEA inhibited PMA and Ca^{2+}-induced PKC activity in a concentration-dependent and SR141617A-sensitive manner when cells were treated with PMA, Ca^{2+} and AEA

for 5 days (Maccarrone *et al.*, 2003b). Thus cannabinoids may couple to both activation and inhibition of PKC although the mechanisms are still unclear.

1.1.4.4 Modulation of NFκB activity

The nuclear factor for immunoglobulin κ chain in B cells (NFκB) family of transcription factors are essential for inflammation, immunity, cell proliferation and apoptosis. NFκB complexes are homo or heterodimers composed of subunits from the multigene family of RelA/p65, c-Rel, NFκB1(p50/p105) and NFκB2(p52/p100). The activation of this family of transcription factors is very complex and thus far three distinct NFκB-activating pathways have been described, all which are based around a cascade of sequentially activated kinases (Viatour *et al.*, 2005, Campbell & Perkins, 2006). The cannonical pathway, which activates NFκB complexes composed of RelA/p65, c-Rel and NFκB1, is outlined in Fig. 1.14. The interest in whether cannabinoids affect NFκB DNA-binding stems from the fact that this transcription factor is involved in interleukin (IL)-2 regulation, one of the factors expressed by activated T lymphocytes which plays a role in T lymphocyte growth and expansion (Nelson, 2004) and cannabinoids have been shown to differentially affect IL-2 production as discussed later (Condie *et al.*, 1996, Ouyang *et al.*, 1998, Jan & Kaminski, 2001, Jan *et al.*, 2002). Equally, both inhibition and activation of NFκB has been demonstrated.

Several studies have demonstrated that cannabinoids inhibit NFκB DNA binding and promotor activity in activated murine leukocytes (Jeon *et al.*, 1996, Ouyang *et al.*, 1998, Herring & Kaminski, 1999, Herring *et al.*, 2001, Kaplan *et al.*, 2005b). The pathways involved in cannabinoid-induced inhibition of NFκB activity have not been extensively studied. However, one study showed that IκBα degradation was inhibited (Herring & Kaminski, 1999) and a role for cAMP inhibition was demonstrated in RAW264.7 murine macrophages (Jeon *et al.*, 1996) but not in murine thymocytes (Herring *et al.*, 2001). However, the role of the cannabinoid receptors was not examined in any of these studies. Cannabinoids were also shown to inhibit TNFα-induced transcriptional activity of NFκB in U373 MG human astrocytoma cells (Juttler *et al.*, 2004) and Jurkats

(Sancho *et al.*, 2003). These effects were suggested to be cannabinoid receptor-independent although the role of the CB_2R in the Jurkats was not fully assessed. One recent study has indicated that 2-AG may be inhibiting NFκB transcriptional activity in a PPAR-γ dependent manner (Rockwell *et al.*, 2006).

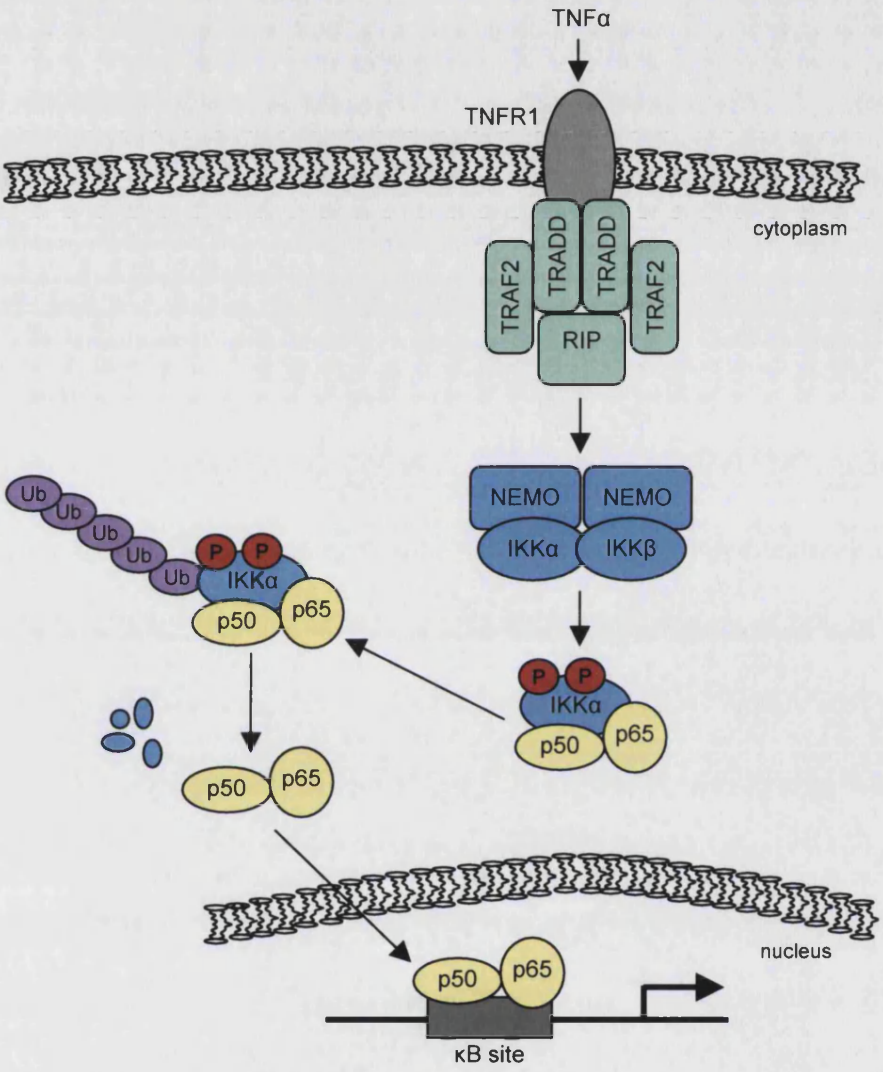


Figure 1.14: **The canonical pathway of NFκB transcription factor activation.** This pathway is activated downstream of pro-inflammatory cytokines such as TNFα. In quiescent cells NFκB, for example p50-p65, is sequestered in the cytoplasm by inhibitory molecules, the inhibitor of NFκB (IκB) proteins, of which IκBα is one. Binding of TNFα to its receptor leads to the recruitment of several adaptor proteins to the membrane. One of these is TNF-receptor-associated factor 2 (TRAF2) which then recruits the IκB kinase (IKK) complex which is composed of IKKα, IKKβ and NFκB essential modulator (NEMO) as well as additional adaptor proteins. Activation of the IKK complex leads to phosphorylation of the IκBα, targeting it for ubiquitination and subsequent degradation via the proteasome pathway. The p50-p65 heterodimer is thus released and translocates to the nucleus where it binds to appropriate κB motifs in DNA (Viatour *et al.*, 2005, Campbell & Perkins, 2006). *Abbreviations:* RIP, receptor-interacting protein; TNFR1, TNFα receptor 1; TRADD, TNF-receptor associated death domain protein.

In contrast, CP55,940 stimulation of HL-60 cells and Δ^9-THC stimulation of dendritic cells led to IκBα degradation, NFκB translocation to the nucleus and upregulation of several genes controlled by NFκB (Derocq *et al.*, 2000, Do *et al.*, 2004). Again, the role of the cannabinoid receptors in NFκB activity specifically was not assessed in these studies.

1.1.4.5 Focal adhesion kinase (FAK) activation

The CB_1R has also been shown to couple to another kinase, focal adhesion kinase (FAK). FAK can be activated by a number of cell surface receptors such as integrins and GPCRs and is found in focal contacts and adhesions as its name suggests. There it acts to regulate the cycle of focal contact formation and disassembly and is thereby involved in the control of cell shape, adhesion and motility, although it has also been linked to the control of cell survival and cell cycle progression (Schlaepfer *et al.*, 2004). The precise mechanism of FAK activation is not yet understood but tyrosine phosphorylation of FAK on various residues plays an important role in regulating the function of the enzyme (Schlaepfer *et al.*, 2004).

Cannabinoids were first linked to FAK activation in rat hippocampal slices (Derkinderen *et al.*, 1996) where cannabinoids stimulated tyrosine phosphorylation of a neuronal isoform of FAK, FAK+, in a PTX-sensitive manner via PKA inhibition. The authors suggested CB_1R-dependent FAK+ activation could have a role in synaptic plasticity. A later study went on to demonstrate a role for both Fyn and other Src family kinases in the pathway between the CB_1R and FAK+ (Derkinderen *et al.*, 2001b). Although CB_1R-dependent FAK+ activation was also shown in another study using rat hippocampal slices (Karanian *et al.*, 2005), HU210, a non-selective cannabinoid agonist, failed to activate FAK+ in differentiated mouse neuroblastoma N1E-115 cells (Zhou & Song, 2002). However, HU210 did stimulate tyrosine phosphorylation of focal adhesion kinase-related non-kinase (FRNK) in a CB_1R-dependent manner via a reduction in intracellular cAMP (Zhou & Song, 2002). FRNK is the C-terminal non-catalytic domain of FAK that has been suggested to be an endogenous regulatory mechanism which negatively regulates the activity

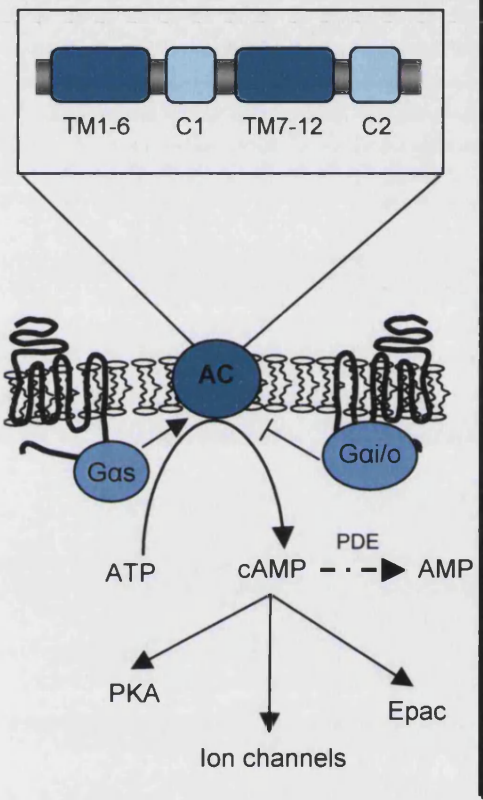
of FAK (Schlaepfer *et al.*, 2004). Furthermore, in a human breast cancer cell line, a stable anandamide analogue, 2-methyl-2'-F-anandamide induced a decrease in FAK tyrosine phosphorylation in a CB_1R-dependent manner (Grimaldi *et al.*, 2006), indicating that the cannabinoids may have different effects on neuronal and non-neuronal isoforms of FAK.

1.1.4.6 Acute modulation of cAMP production

Production of cAMP through the activation of AC can influence a number of cellular functions such as metabolism, cell shape and gene transcription through interaction with a number of downstream molecules, the best known of which is PKA (Fig. 1.15; Kopperud *et al.*, 2003). Inhibition of cAMP production is commonly associated with Gi/o-protein coupled GPCRs and was one of the first cannabinoid receptor-induced signalling pathways to be defined. For instance, Matsuda *et al* (1990) demonstrated that Δ^9-THC and CP55,940 inhibited forskolin-induced cAMP accumulation in CHO cells transfected with the newly cloned CB_1R in a PTX-sensitive manner. Since then both the cannabinoid receptors have been linked to inhibition of forskolin-induced cAMP production in a number of different cell types ranging from neuronal cells to leukocytes (Pertwee, 1997, Kaminski, 1998, Howlett *et al.*, 2002, Demuth & Molleman, 2006).

As well as inhibiting forskolin-induced cAMP production, several reports have linked cannabinoids to increases in forskolin-induced (Glass & Felder, 1997, Felder *et al.*, 1998) and basal cAMP production (Maneuf & Brotchie, 1997, Steffens *et al.*, 2005a). For example, in rat striatum and CHO-CB_1R cells, PTX pre-treatment unmasked a CB_1R-mediated stimulatory effect on cAMP accumulation (Glass & Felder, 1997). It was suggested that this response was mediated through Gs-proteins. Since then there has been further evidence to suggest that the CB_1R can couple to Gs-proteins under certain circumstances (Glass & Felder, 1997, Bonhaus *et al.*, 1998, Jarrahian *et al.*, 2004) and this could explain the increases in cAMP accumulation detected. However, the contrasting effects of cannabinoids on cAMP production could also be attributed to the specific AC isoforms present in different cellular preparations. Thus far

nine distinct isozymes of membrane bound AC have been identified which are differentially regulated as outlined in Fig. 1.15. The effect of cannabinoids on individual AC isoforms has been investigated and showed that AC-I, III, V, VI and VIII where inhibited by, whereas AC-II, IV and VII were stimulated by, CB_1R and CB_2R activation (Rhee *et al.*, 1998). AC-IX inhibition by cannabinoids was very modest.



AC	Regulation by G-protein subunits		Other modulators	
	+	-	+	-
I	Gαs	Gβγ Gαo	PKC Ca^{2+}-CaM	CaMKIV
II	Gαs Gβγ*		PKC	
III	Gαs		PKC Ca^{2+}-CaM	CaMKIV
IV	Gαs Gβγ*			PKC
V	Gαs	Gαi Gβγ	PKC	PKA Ca^{2+}
VI	Gαs	Gαi Gβγ		PKC PKA Ca^{2+}
VII	Gαs Gβγ*		PKC	
VIII	Gαs		Ca^{2+}-CaM	
IX	Gαs			

*, Gβγ-subunit-mediated stimulation of AC is dependent on Gαs co-activation.

Figure 1.15: **Basic outline of G-protein mediated activation of AC.** AC is an integral membrane protein composed of 12 TM segments and 2 large cytoplasmic catalytic loops (C1 and C2). Nine membrane bound isoforms of mammalian AC exist and each is differentially regulated although all can be stimulated by forskolin. Generally AC activity is stimulated by Gαs and inhibited by Gαi subunits whilst Gβγ subunits regulate AC in an isoform-specific manner. AC activity can also be modulated by other factors such as phosphorylation by PKC and Ca^{2+} (Sunahara & Taussig, 2002). Activated AC generates cAMP which goes on to influence a number of different cellular functions, largely through the activation of PKA, although other cAMP effector mechanims do exist (Dremier *et al.*, 2003). *Abbreviations:* CaM, calmodulin; CaMK, calmodulin-dependent kinase; PDE, phosphodiesterase.

1.1.4.7 Modulation of intracellular Ca^{2+} concentration

Cannabinoids appear to stimulate an increase in intracellular Ca^{2+} levels in a number of different cell types via both cannabinoid receptor-dependent and independent mechanisms. For instance, in neuroblastoma x glioma hybrid NG108-15 cells 2-AG increased levels of intracellular Ca^{2+} in a PTX-sensitive manner (Sugiura *et al.*, 1996). Further investigation showed that the response was CB_1R-dependent and sensitive to a PLC inhibitor which suggested the involvement of inositol 1,4,5-triphosphate (IP_3) controlled stores (Demuth & Molleman, 2006). However, the cannabinoid-induced increase in intracellular Ca^{2+} detected in CB_1R transfected CHO cells was not receptor dependent as the same responses were also detected in wild type cells (Felder *et al.*, 1992)

AEA has also been linked to a rise in intracellular Ca^{2+} in endothelial cells in both a CB_1R-dependent and independent manner. For example, in human arterial endothelial cells AEA increased intracellular Ca^{2+} in a SR141716A-sensitive manner (Fimiani *et al.*, 1999). In contrast, in human umbilical vein endothelial cells AEA-induced a rise in intracellular Ca^{2+} which was only partly blocked by SR141716A and insensitive to PTX (Mombouli *et al.*, 1999). In this study it was suggested that the Ca^{2+} was released from caffeine sensitive stores. The mobilisation of cytosolic Ca^{2+} in endothelial cells has been suggested to play a role in the endothelium-dependent and nitric oxide mediated vasodilator actions of AEA.

An early study using CB_2R transfected CHO cells suggested that the receptor did not couple to intracellular Ca^{2+} mobilisation (Slipetz *et al.*, 1995). However, the CB_2R has now been linked to a rise in intracellular Ca^{2+} in calf pulmonary endothelial cells where AEA induced an increase in intracellular Ca^{2+} from IP_3-sensitive stores through the activation of PLC (Zoratti *et al.*, 2003) and HL-60 cells (Sugiura *et al.*, 2000).

1.1.4.8 Ion channel effects

Cannabinioids can activate A-type and inwardly rectifying K^+ channels and inhibit N-type and P/Q-type voltage gated Ca^{2+} channels as well as D-type K^+ channels downstream of the CB_1R (Howlett *et al.*, 2002, Pertwee & Ross, 2002, van der Stelt & Di Marzo, 2005, Demuth & Molleman, 2006). The CB_2R is not believed to modulate ion channel function.

In addition to CB_1R-mediated effects, AEA has also been shown to directly interact with a number of neurotransmitter-gated ion channels such as NMDA, 5-HT_3 and α7-nicotinic acetylcholine receptors. For instance, AEA augmented the NMDA-induced current and acetylcholine currents were inhibited (van der Stelt & Di Marzo, 2005). Several cannabinoid agonists such as AEA and WIN55,212-2 have been shown to inhibit 5-HT–induced currents through the 5-HT_{3A} receptor, a ligand-gated ion channel (Barann *et al.*, 2002) as mentioned earlier. Furthermore, AEA can also directly inhibit T- and L-type Ca^{2+} channels, Shaker-related K^+ channels, the background K^+ channel TASK-1 and voltage gated Na^+ channels (van der Stelt & Di Marzo, 2005, Demuth & Molleman, 2006).

1.2 T lymphocytes

The immune system is divided into two branches: innate immunity and adaptative immunity. The innate immune response is the first response to any pathogen and defends against it in a non-specific manner. For instance, neutrophils and macrophages are part of the innate immune response. The adaptive immune response is the response of antigen-specific lymphocytes to antigen. T lymphocytes form a major part of the adaptive immune response and are especially important in developing immunological memory (Janeway *et al.*, 1999).

1.2.1 The T cell antigen receptor (TCR)

In general T lymphocytes only recognise short peptide antigen bound to major histocompatability complex (MHC) molecules which is presented by cells known

as antigen-presenting cells (APCs). There are two classes of MHC molecules. Class I molecules are found on virtually all cells and present peptides derived from intracellular pathogens such as viruses. In contrast, class II MHC molecule expression is restricted to macrophages, dendritic cells and B lymphocytes which are said to be professional APCs. This type of MHC molecule presents peptides derived from pathogens residing extracellularly which have been taken into cells via endocytosis. T lymphocytes are said to be MHC restricted because their receptors are specific for both a particular MHC allele and the antigen peptide it binds (Hennecke & Wiley, 2001).

It is the TCR on the T lymphocytes which recognises the peptide/MHC complex. The TCR is composed of two polypeptide chains which in most T lymphocytes are the α and β-chains. However, there is a subset of T lymphocytes which express a TCR composed of γ and δ-chains. These chains are composed of a N-terminal variable region, parts of which form the antigen binding site, and a C-terminal constant region which is attached to the TM region. It is differences in the amino acid sequence of the variable region that creates the enormous variety of binding sites that are specific for particular peptide/MHC molecule complexes. This diversity is generated by gene rearrangement in a process referred to as somatic recombination. The αβ and γδ dimers are associated with cluster of differentiation (CD)3, a complex itself composed of γ, δ and ζ-chains, and TCR ζ-chains which are important in T lymphocyte activation as they relay signalling into the cell. All together these form the TCR complex. Activation of the TCR triggers a cascade of intracellular biochemical events which eventually lead to proliferation and effector functions which are discussed in further detail below (Janeway *et al.*, 1999).

1.2.2 T lymphocyte subsets

T lymphocytes can be subdivided into two main classes. Cytotoxic T lymphocytes, which are distinguishable by the expression of the cell surface glycoprotein, CD8, act to kill infected cells and thereby help to prevent the spread of infection. When exposed to infected cells, they release cytotoxins such as perforin, which form pores in the target cells' plasma membrane eventually

lysing it, and granzymes, proteases which activate apoptosis. They also express CD95L (also known as Fas ligand), which upon binding its receptor (CD95) on the target cell induces apoptosis (Janeway *et al.*, 1999). $CD8^+$ T lymphocytes recognise peptide presented by cells bearing MHC class I molecules. Helper T lymphocytes (Th), which express CD4, are involved in co-ordinating the immune response. The two main types of Th lymphocytes are Th1 and Th2 which secrete different profiles of cytokines and have distinct functions, although additional subsets have been described. Th1 cells secrete interferon (IFN)γ and are thus involved in activating macrophages. Th2 cells secrete IL-4 and IL-5 which activate B lymphocytes and also IL-10 which inhibits macrophage function. $CD4^+$ T lymphocytes recognise antigen when presented by MHC class II molecules (Janeway *et al.*, 1999, Reinhardt *et al.*, 2006).

In addition there are also regulatory T lymphocytes and natural killer T lymphocytes. Regulatory T lymphocytes, sometimes also referred to as suppressor T lymphocytes, actively suppress activation of the immune system and prevent pathological self-reactivity or autoimmune disease. Different subsets exist, including $CD4^+$ cells that also express high levels of the IL-2 receptor α-chain (CD25). The transcription factor forkhead box p3 is required for the development and function of these $CD4^+CD25^+$ regulatory T lymphocytes (Thompson & Powrie, 2004). Natural killer T lymphocytes are a heterogenous group of T lymphocytes that share properties of both T lymphocytes and natural killer (NK) cells. They are reactive to glycolipid when presented by CD1d, an atypcial MHC class I molecule, and are potent cytokine producers. They express an invariant TCR α-chain as well as NK cell markers and can be $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ (Godfrey *et al.*, 2004).

There is also a minor population of T lymphocytes which, unlike the vast majority of T lymphocytes, expresses a TCR composed of γ and δ-chains rather than α and β-chains. They can exert both cytotoxic effects as well as producing cytokines and are capable of regulating various responses of the innate immune system, some of which in turn affect adaptive immune responses. Thus they have been described as a bridge between the innate and adaptive immune responses. They are different from αβ T lymphocytes in that they recognize

soluble protein and non-protein antigens and that they are not MHC restricted. Most are $CD4^-CD8^-$ and although they represent only 1-5% of T lymphocytes in peripheral lymphoid tissues, they can represent up to 50% of the T lymphocyte population of epithelial-rich tissues such as the intestine (Carding & Egan, 2002).

1.2.3 T lymphocyte development

T lymphocytes are derived from bone marrow haematopoietic stem cells that enter and populate the thymus. The specific identity of these progenitors is still unclear. Immature T lymphocytes (also called thymocytes) go through several distinct phenotypic stages before they leave the thymus as mature $CD4^+$ or $CD8^+$ T lymphocytes. These include the gene rearrangments that produce the TCR and positive and negative selection which determines the T lymphocyte repertoire (Fig. 1.16; Germain, 2002). These naïve T lymphocytes then leave the thymus and recirculate through the blood, homing to secondary lymphoid organs such as the lymph nodes. Inside these, the T lymphocytes screen APCs for peptide/MHC complexes and those that recognise peptide/MHC molecules are activated and will divide and differentiate into effector T lymphocytes. Each T lymphocyte that is activated by a particular antigen gives rise to a clonal population of cells all expressing the same TCR. This process is referred to as clonal selection. Activated T lymphocytes eventually leave the secondary lymphoid organs to accumulate at sites of inflammation or other effector sites (Stein & Nombela-Arrieta, 2005). Importantly, some T lymphocytes also become memory cells which are long-lived antigen-specific T lymphocytes that become activated during a secondary immune response to a pathogen already encountered. They retain the TCR affinity of the originally activated T lymphocyte and are used to act later as effector cells (Lanzavecchia & Sallusto, 2005).

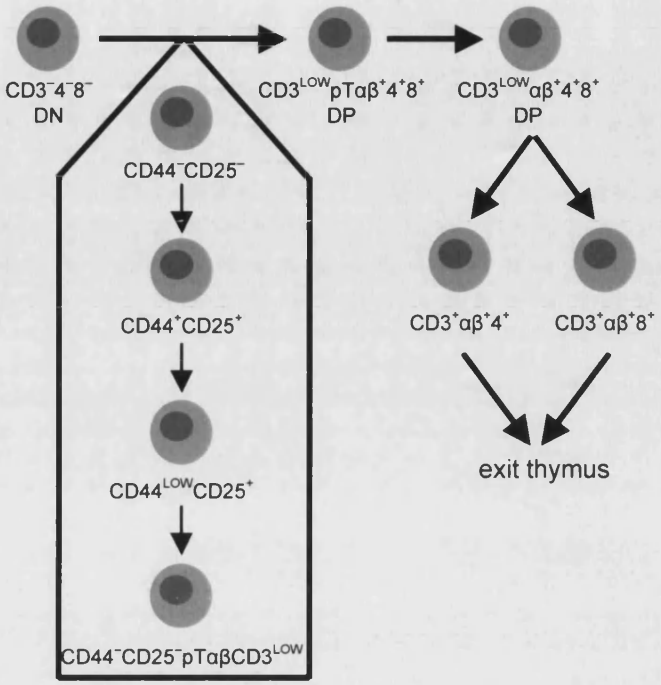


Figure 1.16: **Development of αβ T lymphocytes in the thymus.** Haematopoietic stem cells enter the thymus and interactions with the thymic stroma triggers differentiation and proliferation such that double negative (DN) thymocytes are generated. These cells are committed to the T lymphocyte lineage but still lack all the major markers of mature T lymphocyte, namely CD3, CD4 and CD8. These cells can still develop along the αβ or γδ pathway although the focus here is on αβ T lymphocytes. These DN thymocytes cells at first express CD44, an adhesion molecule, but not CD25 and then differentiate to express CD25 as well. As they continue to mature they start to loose CD44 expression and it is in these $CD44^{LOW}CD25^{+}$ cells that rearrangement of the TCR β-chain gene occurs. The cells that successfully express a β-chain lose CD25 and the β-chain pairs with a surrogate α-chain which is referred to as the pre-T-cell α (pTα) and low levels of CD3 molecules. Once this complex is expressed on the surface and the cell cycle is triggered causing the cells to proliferate, CD25 is lost, β-chain rearrangement is arrested and cells begin to express CD4 and CD8 thus becoming double positive (DP) thymocytes. Once these stop proliferating the TCR α-chain gene rearranges, producing the complete αβ TCR although it is still only expressed at low levels. Cells then undergo negative and positive selection. Negative selection determines whether the T lymphocyte recognises self antigens with high affinity, and if they do, they receive signals to apoptose, thereby eliminating autoreactive T lymphocytes. Positive selection determines whether the T lymphocyte is able to recognise and bind self-MHC molecules and only those cells which bind the MHC complex with enough affinity receive survival signals. Those cells that survive then differentiate and mature into single positive, $CD4^{+}$ or $CD8^{+}$, lymphocytes which can exit the thymus (Germain, 2002).

1.2.4 T lymphocyte activation

Naïve T lymphocyte activation involves two signals. First the TCR interacts with specific MHC/peptide complex presented by APCs. It is largely the CD3 and ζ-chains within the TCR complex that are responsible for transducing signal into the cells via immunoreceptor tyrosine-based activation motifs (ITAMs). These conserved motifs act as tyrosine kinase substrates and once phosphorylated, as binding sites for other kinases. In this way the TCR complex is coupled to a wide range of kinases and adaptor proteins which intiate signals that bring about T lymphocyte activation. CD4 is important for stimulating molecular clustering at the immunological synapse and for stabilising cell-cell interactions such that the T lymphocyte can interact with the APC for a sufficient length of time for activation to occur. Further stabilisation occurs by adhesion molecules such as leukocyte function associated antigen-1 (LFA-1) which binds intracellular adhesion molecule-1 (ICAM-1) on the APC (Zhang & Samelson, 2000, Samelson, 2002).

Stimulation of the TCR alone is not sufficient to induce activation. A second, co-stimulatory, signal is required to amplify signals from the TCR and prevent the T lymphocyte from becoming functionally inert or anergic. CD28 on the naïve T lymphocyte interacts with B7 molecules on the APC and this is necessary to induce IL-2 transcription, CD25 expression, entry into the cell cycle and enhance cell survival (Kroczek *et al.*, 2004). Activation is also negatively regulated in order to limit the T lymphocyte response. One of the main proteins involved is the co-stimulatory molecule, cytotoxic T lymphocyte antigen-4 (CTLA-4). Unlike CD28, which is constitutively expressed, CTLA-4 is upregulated on activated T lymphocytes (Kroczek *et al.*, 2004).

Once active, naïve T lymphocytes differentiate into effector cells. $CD4^+$ T lymphocytes differentiate into Th1 or Th2 cells and this is dependent on the production of IL-12 (Th1) and IL-10 (Th2) by dendritic cells as well as the balance of other cytokines such as IL-4 and IFNγ in the local environment. Activated $CD8^+$ T lymphocytes differentiate into cytotoxic T lymphocytes. In

both instances it is IL-2, which acts in an autocrine or paracrine fashion, which stimulates their proliferation (Janeway *et al.*, 1999).

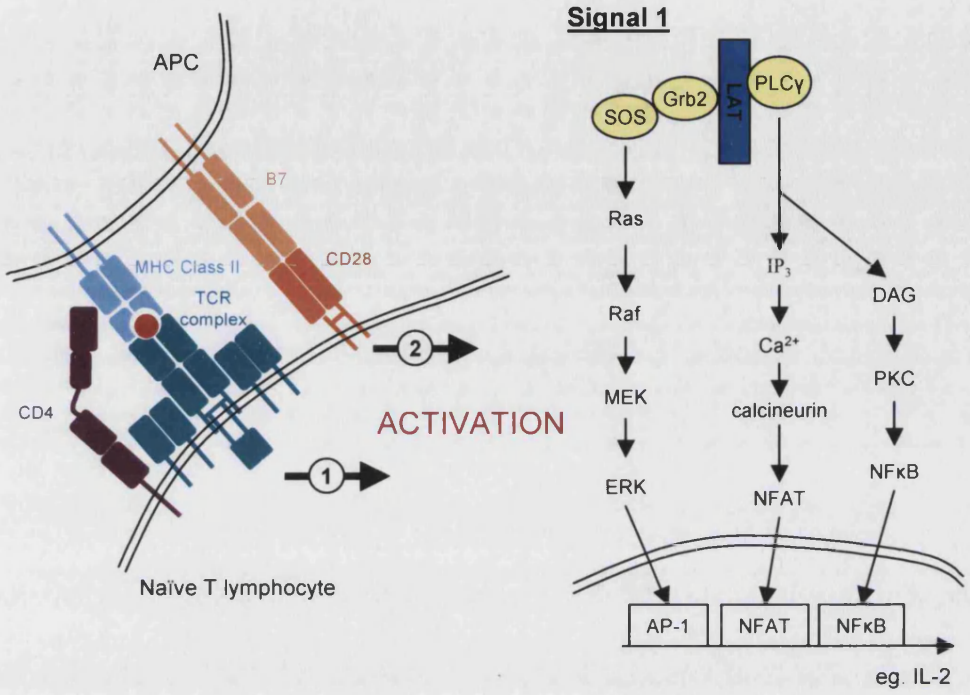


Figure 1.17: **Naïve T lymphocyte activation involves two signals.** First the TCR recognises antigen presented via an MHC molecule. In this example the cell is $CD4^+$ and therefore binds MHC class II molecules. This initiates the phosphorylation of ITAMs found within the CD3 subunits and ζ-chain of the TCR complex. Lymphocyte-specific protein tyrosine kinase (Lck) is the predominant kinase involved in this. Lck is a Src family kinase and interacts with the cytoplasmic tail of CD4 and CD8 such that when they bind MHC molecules Lck is brought into the proximity of the TCR complex, thereby facilitating ITAM phosphorylation. Phosphorylated ITAMs then provide docking sites for a 70kD TCR ζ-chain-associated protein kinase (ZAP-70), a Syk family protein tyrosine kinase, via its SH2 domains. Upon recruitment, ZAP-70 is phosphorylated and activated by the already functional Lck. One of the downstream effectors of ZAP-70 is linker for activation of T cells (LAT). This adaptor protein can associate with a great number of proteins and connects several second messenger pathways, as shown above, to increased activity of transcription factors such as activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and NFκB and thereby expression of new proteins such as CD25 and IL-2. A second signal is also required for T lymphocyte activation where CD28 interacts with CD80 (B7.1) or CD86 (B7.2) on professional APCs. One of the first events in CD28 activation is its phosphorylation by Lck, creating a SH2-binding motif for a number of proteins including the p85 adaptor subunit of class IB PI3Ks. PI3K, through its downstream effector PKB, plays an important role in the differentiation, survival, cell-cycle entry and growth stimulated by T lymphocyte activation (Zhang & Samelson, 2000, Samelson, 2002, Okkenhaug & Vanhaesebroeck, 2003, Matthews & Cantrell, 2006).

1.2.5 Migration

1.2.5.1 Recruitment of T lymphocytes into secondary lymphoid organs and inflamed tissue

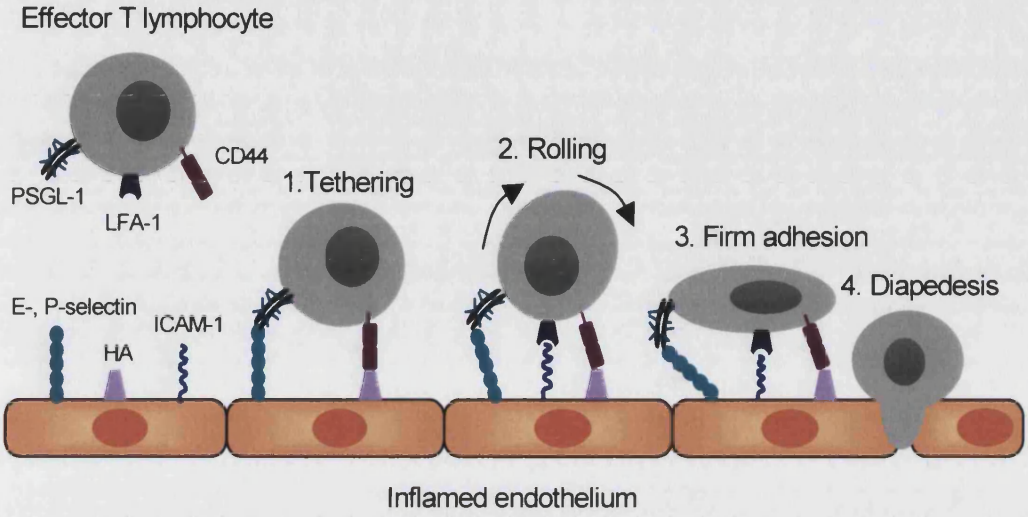


Figure 1.18: **Recruitment of effector T lymphocytes from blood into a site of inflammation.** Firstly the effector T lymphocyte is tethered to the endothelium via adhesion molecules such as selectins and low affinity integrins. In this example, P-selectin glycoprotein ligand-1 (PSGL-1) and CD44 binding to their respective ligands initialises tethering to the endothelium. Due to the shear flow in the vessel these initial interactions between the T lymphocytes and the endothelial cells are only transient and so the T lymphocyte appears to roll along the endothelium. Chemoattractants, such as chemokines, bound to the surface of the endothelium via glycosaminoglycans activate GPCRs on the T lymphocyte which in turn triggers increases in affinity and avidity of integrins, such as $\alpha_L\beta_2$ (also known as LFA-1), thereby allowing the leukocytes to adhere more firmly to the endothelial cells. The T lymphocyte can then migrate through the vessel wall into the underlying tissue via a process known as diapedesis. The main route of diapedesis noted *in vitro* and *in vivo* is paracellular and involves the transient disassembly of endothelial junctions followed by penetration through the underlying basement membrane. Although this process is essentially the same for all leukocytes, including the various subtypes of T lymphocytes, specificity is ensured such that the correct types of leukocytes are recruited under any given circumstances. This specificity is achieved by the selective expression of adhesion molecule pairs and chemoattractants and their receptors on leukocytes and endothelial cells, factors which can be influenced by cellular activation status and inflammatory mediators. Thus, for instance, naïve T lymphocytes leaving the thymus express molecules which mean they respond poorly to inflammatory signals but will migrate efficiently to secondary lymph tissues where they transmigrate through specialized high endothelial venules and endothelial cells in peripheral tissues will permit little/no leukocyte binding unless exposed to inflammatory mediators (Johnston & Butcher, 2002, Steeber *et al.*, 2005, Luster *et al.*, 2005). Adapted from Steeber *et al*, 2005. *Abbreviations*: HA, hyaluronan.

The migration of T lymphocytes is important in T lymphocyte development, immunosurveillance and the immune response. As already mentioned naïve T lymphocytes circulate through the blood until they reach secondary lymph tissues where they will encounter antigen and are transformed into effector cells. These cells then recirculate through the blood until they reach the site of inflammation or non-lymphoid tissues such as skin or gut. The process by which leukocytes, including T lymphocytes, are recruited from the blood to lymphoid and peripheral tissues is outlined in Fig. 1.18. Briefly, interactions mediated by surface adhesion molecules cause leukocytes to tether to and then roll along the endothelium. Chemoattractants, such as chemokines presented on the endothelial cell surface, trigger increases in integrin affinity and avidity thereby inducing firmer adhesion of the leukocytes to the endothelial cells. The leukocytes can then transmigrate through the vascular wall into the extravascular tissue where they are then further guided by chemokines and other chemoattractants into specific tissue environments (Johnston & Butcher, 2002, Luster *et al.*, 2005, Steeber *et al.*, 2005).

1.2.5.2 Types of chemoattractants and modes of migration

The different types of chemoattractant agents known to date and various modes of migration are outlined in Fig. 1.19. Essentially the largest group of chemoattractants is that of the chemokines which are small peptides that signal through GPCRs. Some chemokines, such as CXCL12, are mainly homeostatic whilst others play more of a role in inflammation. However the division of the chemokines into homeostatic and inflammatory ones is not categorical. Chemoattractants provide directional cues for the movement of cells and abarrent recruitment of cells in inflammatory and autoimmune diseases can be associated with upregulation of chemoattractants and their receptors. Cells are capable of migrating in many different ways. The term chemotaxis refers to directed cell migration up a concentration gradient of a soluble chemoattractant, whereas chemofugetaxis is movement down a concentration gradient. *In vivo* chemokines exist not only as soluble peptides but can also be surface bound. Cell migration towards surface bound chemoattractants is referred to as haptotaxis and repulsion

from them is haptorepulsion. Finally, chemokinesis refers to random cell movement in the absence of any chemoattractant gradient.

Chemoattractant	
Chemokines	CXCL8, CXCL9, CXCL10, CXCL12, CXCL13, CXCL16, CCL1, CCL2, CCL11, CCL17, CCL19, CCL21, CCL22, CCL25, CCL28
Lipids	S1P, LPA, PGD_2, LTB_4
Microbial and complement products & small peptides	Defensins, fMLP, C5a
Others	IL-16, 5-HT

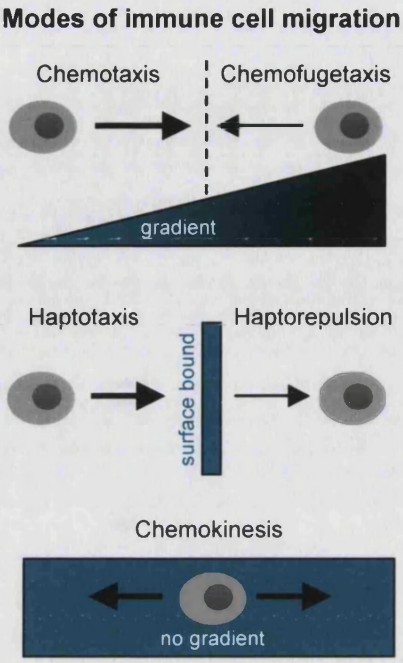


Figure 1.19: **Types of chemoattractants and the various types of immune cell migration.** Adapted from Kim, 2005. *Abbreviations*: C5a, activated complement component 5; LTB_4, leukotriene B4; S1P, sphingosine-1-phosphate.

1.2.5.3 Steps involved in directed cell migration

Migration of any cell is itself made up of several steps. The first response to a chemoattractant is for the cell to polarize and extent protrusions or pseudopodia in the direction of migration. A dominant pseudopod then adheres to the substratum, for instance the extracellular matrix, which stabilises it. These adhesions also serve as tractions sites for migration. Net forward movement of the cell is achieved when the cell body contracts and releases attachment sites at the rear. In slow moving cells such as fibroblasts these steps are very distinct but the stages are less obvious in faster moving cell types such as leukocytes (Ridley *et al.*, 2003, Chodniewicz & Klemke, 2004).

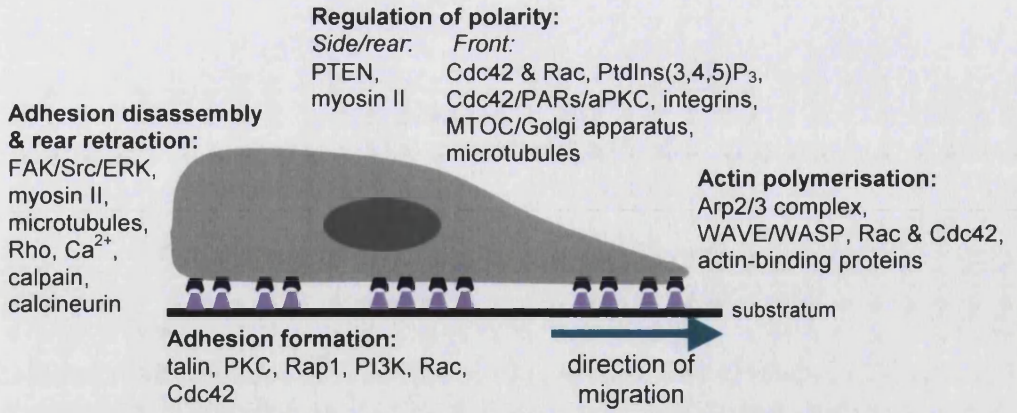


Figure 1.20: **Basic outline of a migrating cell and some of the molecules involved in the different steps of directed migration.** Directed cell migration can be thought of as a cyclical process which starts with cell polarisation. Establishing and maintaining polarity in response to the external stimulus is mediated by several interlinked feeback loops. This is then followed by the extension of protrusions and adhesion formation at the front or leading edge of the cell and is completed when the rear of the cell detaches from the substratum and retracts. Many signalling molecules have been implicated in these various stages, some of which are shown. For example, the Rho GTPase Cdc42 is key in regulating polarity and is mainly active at the leading edge. It is involved in regulating where lamellipodia form and microtubule-organising centre (MTOC) and Golgi apparatus localisation. Where another member of the Rho GTPase family, Rac, is active in the cell is involved in defining where actin polymerisation occurs and thus is important in maintaing directional protrusion. Several feedback loops exist to help maintain active Rac at the leading edge. In contrast, a third RhoA GTPase, Rho, is active at the rear of the cell and is involved in controlling rear retraction (Fukata *et al.*, 2003, Ridley *et al.*, 2003). Adapted from Ridley *et al*, 2003. *Abbreviations*: Arp2/3, actin-related protein 2/3; WASP, Wiskott-Aldrich syndrome protein; WAVE, WASP-family verprolin homologuos protein.

1.2.5.3.1 Polarisation

The term polarisation refers to the fact that the molecular processes at the front and the rear of the cell are different and is induced by chemoattractants such as chemokines. Morphological polarity is characterised by the formation of a dominant pseudopod in the direction of migration. The front of the cells thus becomes the leading edge and the rear is the trailing edge, sometimes also referred to as the uropod. Notably, cell polarity is also defined in terms of the positioning of the nucleus and reorientation of the MTOC and Golgi apparatus towards the leading edge, although this is less important in fast moving cells such as leukocytes (Ridley *et al.*, 2003, Vicente-Manzanares *et al.*, 2005).

Cells not only exhibit morphological polarity but also in terms of intracellular signalling molecules. For instance, it is important in directional sensing, which is the ability of the cell to detect even a very shallow chemoattractant gradient and generate an internal amplied response. One of the first polarised responses in many leukocytes is the accumulation of the PtdIns(3,4,5)P_3, at the leading edge. This is caused by the activation of PI3K at the leading edge and feedback loops that amplify PtdIns(3,4,5)P_3 synthesis at the front of the cell and control its degradation at the sides and back of the cell. PtdIns(3,4,5)P_3 itself is involved in protrusion formation and when a new pseudopod is formed this occurs preferentially at a site with elevated levels of PtdIns(3,4,5)P_3 (Ridley *et al.*, 2003, Van Haastert & Devreotes, 2004, Procko & McColl, 2005).

1.2.5.3.2 Protrusion and adhesion formation

Cells can extend different types of pseudopods. For instance, lamellipodia are large, broad protrusions that may serve as the main basis for directional migration whereas filopodia are spike-like protrusions which may instead act as sensors which explore the extracellular environment. Indeed, in the absence of a directional cue cells can move randomly, sending out pseudopodia in several directions. When they encounter a chemoattractant gradient one dominant pseudopod is extended at what becomes the leading edge of the cell.

These protrusions are driven by actin polymerisation. Actin is a structural protein which exists as monomers known as globular actin (G-actin) and polymerises into filaments when it is known as filamentous actin (F-actin). Actin filaments are intrinsically polarized with fast-growing 'barbed' ends and slow-growing 'pointed' ends (Fig. 1.21). In lamellipodia the actin exists as a branched network of short filaments and its polymerisation is mediated by the Arp2/3 complex which binds the sides or tip of a pre-existing actin filament and promotes the extension of a new filament. The Arp2/3 complex is localised by WASP/WAVE family members which are in turn activated by Cdc42 and Rac. In contrast, the actin in filopodia is organised in long parallel bundles. Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins are found in the tip of filopodia and are involved in catalysing elongation of newly formed

filaments (Ridley *et al.*, 2003, Revenu *et al.*, 2004, Vicente-Manzanares *et al.*, 2005).

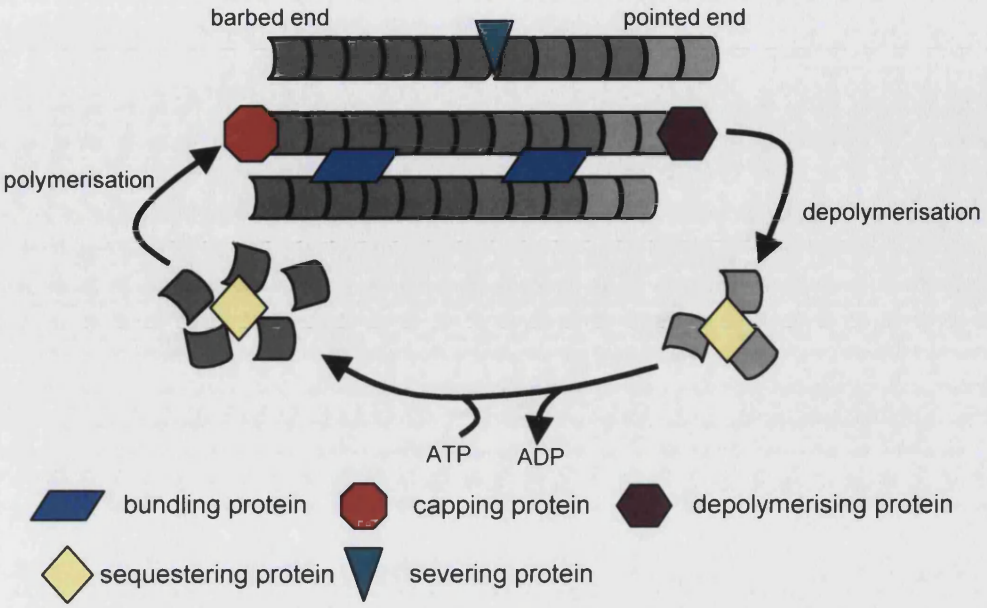


Figure 1.21: **Structure and dynamics of actin filaments.** G-actin monomers can associate to form helical filaments which consist of a fast-growing barbed and a slow-growing pointed end. When the concentrations of G-actin and F-actin are at equilibrium, there is a loss of actin monomers from the pointed end and an equal addition of monomers at the barbed end such that there is a flow of actin subunits through the filament. This is often referred to as treadmilling. Actin monomers can bind either ADP or ATP and the addition of ATP-actin monomers is favoured at the barbed end. As the ATP is hydrolysed to ADP, ADP-actin monomers are released from the pointed end. Several proteins can regulate the rate and organisation of actin polymerisation. Bundling proteins are implicated in the organisation of actin filaments into bundles in structures such as filopodia. For example, in filopodia, fascin is found along the length of the protrusion wheras α-actinin is found at the rear of the protrusion. Capping proteins, such as gelsolin, block actin polymerisation at the barbed ends, terminating filament elongation and are important in maintaining filament length in branched networks such as lamellipodia. Sequestering proteins can control the availability of activated actin monomers. Profilin not only binds actin monmers but also catalyses the exchange of ADP for ATP. Members of the actin-depolymerising factor (ADF)/cofilin family of proteins not only act as severing proteins, creating free barbed ends, but also promote depolymerisation of free filament pointed ends and thus play an important role in controlling the turnover of actin filaments. Finally, actin bundles and branched networks are also linked to the plasma membrane via actin-membrane-protein linkers such as members of the ezrin/radixin/moesin family and this link is important in the formation and maintenance of protrusions. All of these actin-binding proteins themselves are also under regulation by different proteins. (Ridley *et al.*, 2003, Revenu *et al.*, 2004, Vicente-Manzanares *et al.*, 2005). Adapted from Revenu *et al*, 2004.

Although the protrusion of the leading pseudopod is key in migration additional steps are involved in achieving cell migration. The adhesion of the leading pseudopod to the underlying substratum is very important and it has been shown that pseudopodia that do not establish adhesive contacts retract back into the cell

body. TM receptors of the integrin family are key in the formation of adhesion complexes. They are heterodimeric receptors composed of non-covalently associated α and β subunits and the group includes α_4 integrins such as $\alpha_4\beta_1$ (also known as very late antigen-4) and β_2 integrins such as $\alpha_L\beta_2$. Integrins are largely maintained in a low affinity conformational state but, as mentioned earlier, chemoattractants can signal to activate or functionally upregulate integrins (Ridley *et al.*, 2003, Laudanna & Alon, 2006). Activated integrins preferentially localise at the leading edge of cells. Binding of ligands to the large extracellular binding domain of the integrins induces conformational changes and clustering which initiates intracellular signalling, the effects of which include regulation of adhesion site formation and strengthening. Just as cells are capable of producing different types of protrusions, different types of adhesions also exist and although integrins are an integral part of these adhesions they also contain many other proteins including structural and signalling components. In non-migratory and very slow moving cells large integrin clusters called focal adhesions are formed and tightly adhere the cells to the substratum. In other cells smaller adhesions, known as focal complexes form at the leading edge. However, many rapidly migrating cells such as leukocytes have very few integrin clusters and therefore appear to glide over the substratum (Ridley *et al.*, 2003).

1.2.5.3.3 Achieving net forward movement of the cell

Formation of adhesive contact is necessary not only to stabilise the pseudopod but also to generate the contractile force to pull the cell forward. Myosin II has been shown to link these adhesion complexes with actin filaments to produce the contractile force used to move the cell forward. Myosin II activity is controlled by phosphorylation of myosin light-chain (MLC). Phosphorylation of MCL is caused by MLC kinase (MLCK), which is regulated by Ca^{2+} and several kinases, and Rho kinase (ROCK), which as its name suggests is regulated by RhoA, and phosphorylation leads to increased contractility (Ridley *et al.*, 2003, Chodniewicz & Klemke, 2004, Vicente-Manzanares *et al.*, 2005).

In order for the rear of the cell to retract and the cell to move forward adhesions at the trailing edge must disassemble. Currently it is still unclear exactly how

adhesion disassembly occurs but several mechanisms have been implicated. For example, the high tension exerted on the rear adhesions may contribute to detachment. Equally, modulation of integrin affinity and the cytosolic components of adhesion has been suggested to play a role. For instance, these could be cleaved by the protease calpain. The tyrosine kinases Src and FAK as well as intracellular Ca^{2+} levels have also been associated with disassembly of rear end adhesions (Ridley *et al.*, 2003, Kirfel *et al.*, 2004, Vicente-Manzanares *et al.*, 2005).

1.3 Cannabinoid functions in the immune system

One of the first reports of cannabinoids having any effect on the immune system came from the 1970s when it was suggested that cannabis use was associated with an increased incidence of viral infection (Cabral & Dove Pettit, 1998). Although many studies have shown that cannabinoids are immunosuppressive, data also suggests that cannabinoids can exert pro as well as anti-inflammatory actions and may be important in the control of immune reactions (Croxford & Yamamura, 2005). It is also noteworthy that often the inhibitory effects of cannabinoids occur in the micromolar range which may not be physiological (Croxford & Yamamura, 2005). However, the potential therapeutic use of cannabinoids in inflammatory diseases continues to be studied (Di Marzo *et al.*, 2004, Klein, 2005). Thus, whilst the exact role of the cannabinoids in the immune system remains unclear they are currently described as immunomodulatory.

Just as cannabinoids affect a number of intracellular signalling pathways, they also affect a number of different immune cell functions such as cytokine production and migration as indicated in Fig. 1.22. Only some of these will be discussed here with emphasis on those relevant to T lymphocyte function. The CB_2R is generally considered the 'immune' cannabinoid receptor and is suggested to be involved in some of the immunumodulatory functions of the cannabinoids, although cannabinoid receptor-independent effects have also been noted. A CB_2R knockout mouse has been generated and is helping to establish

the role of the receptor in cannabinoid-induced immune system modulation (Buckley *et al.*, 2000, Chuchawankul *et al.*, 2004).

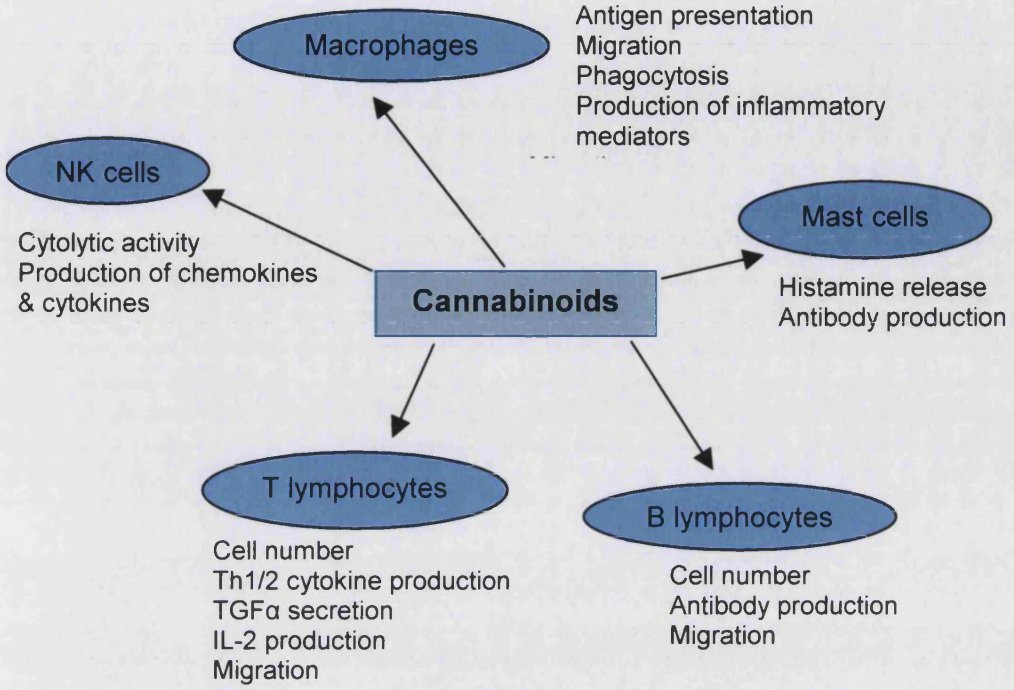


Figure 1.22: **Schematic diagram to represent the effects of cannabinoids on different immune subpopulations.** Adapted from Croxford & Yamamura, 2005. *Abbreviations*: TGF, transforming growth factor.

1.3.1 Modulation of cell migration

Cannabinoids have been reported to affect immune cell migration, although it is noteworthy that they also affect migration of non-immune cells. Firstly, they have been shown to affect spontaneous migration and several studies have suggested that 2-AG acts as a chemoattractant for immune cells including HL-60 cells differentiated into macrophage-like cells (Kishimoto *et al.*, 2003), CB_2R-expressing myeloid precursor cells (Jorda *et al.*, 2003), B lymphocytes (Jorda *et al.*, 2002, Rayman *et al.*, 2004) and eosinophils (Oka *et al.*, 2004). When receptor involvement was studied in immune cell migration it was shown that CB_2R was involved (Jorda *et al.*, 2003, Oka *et al.*, 2004, Rayman *et al.*, 2004). Although 2-AG is generally considered to induce migration, results using other cannabinoids have been mixed. Cannabinoids may also alter ligand-induced migration, although again the effects may be ligand and cell-type specific. For

instance, CP55,940 inhibited formyl-metionyl-leucine-phenylalanine (fMLP)-induced rat peritoneal macrophage migration (Sacerdote *et al.*, 2000), yet AEA did not alter fMLP-induced migration of human neutrophil granulocytes (Joseph *et al.*, 2004). Thus, cannabinoid effects on both basal and ligand-induced migration appear to be both ligand and cell-type specific. Examples of how cannabinoids affect immune cell migration are outlined in Table 2.

Cell type	Migration studied	Cannabinoid	Effect	References
Murine B lymphocytes	basal	2-AG	induced	Jorda *et al.*, 2002
Myeloid precursor cells	basal	2-AG, AEA	induced	Jorda *et al.*, 2002, Jorda *et al.*, 2003, Jorda *et al.*, 2004
		PEA, CP55,940, WIN55,212-2, cannabinol, cannabidiol, Δ^9-THC	no effect	
HL-60 (undifferentiated)	basal	CP55,940	induced	Derocq *et al.*, 2000
		2-AG	no effect	Kishimoto *et al.*, 2003
HL-60 (differentiated into macrophage like cells)	basal	2-AG, noladin ether, CP55,940, WIN55,212-2	induced	Kishimoto *et al.*, 2003
		AEA	no effect	
Rat peritoneal macrophages	fMLP-induced	CP55,940	inhibited	Sacerdote *et al.*, 2000
Murine macrophages	CCL2-induced	Δ^9-THC	inhibited	Steffens *et al.*, 2005b
	fMLP-induced	cannabidiol	inhibited	Sacerdote *et al.*, 2005
Human monocytes	basal	2-AG	induced	Kishimoto *et al.*, 2003
Human neutrophil granulocytes	fMLP-induced	AEA	no effect	Joseph *et al.*, 2004
	basal	AEA	no effect	
Human neutrophils	basal	2-AG	no effect	Oka *et al.*, 2004
Human eosinophils	basal	2-AG, noladin ether	induced	Oka *et al.*, 2004
		AEA	no effect	
BV-2 murine microglial cells	basal	AEA, 2-AG, cannabidiol, abn-cannabidiol, ACPA	induced	Franklin *et al.*, 2003, Walter *et al.*, 2003, Franklin & Stella, 2003

Table 2: **Cannabinoid effects on immune cell migration.** Examples of how cannabinoids affect migration in a number of different immune cell types.

In vivo, cannabinoids may also be able to affect cell migration indirectly by altering the production of chemokines or chemokine receptor expression. For example, in mice treated with thioglycollate broth to induce peritonitis it was found that two synthetic cannabinoids HU210 and WIN55,212-2 blocked the migration of neutrophils into the peritoneal cavity and that this effect was caused by a delay in the production of the chemokines CXCL1 and CXCL2/3 (Smith *et al.*, 2001). It has also been shown *in vitro* that Δ^9-THC can inhibit TNFα-induced increases in CCR2 mRNA expression in mouse splenocytes (Steffens *et al.*, 2005b). Cannabinoid effects on cytokine and chemokine production will be further discussed below.

1.3.2 Modulation of cytokine and chemokine production

1.3.2.1 General

The first evidence that cannabinoid could modulate cytokine production came came from experiments carried out in the 1980s (Klein, 2005). Murine splencoytes treated with Δ^9-THC produced less IFN when subsequently stimulated with PHA, concanavalin A or lipopolysaccharide (LPS) compared to controls (Blanchard *et al.*, 1986). Mice which were chronically exposed to Δ^9-THC *in vivo* also demonstrated suppressed IFN response to these mitogens *in vitro* (Blanchard *et al.*, 1986).

Cannabinoids have been shown to suppress the production of a number of cytokines and chemokines. Since those early studies, decreases in levels of IFNγ production have also been described in studies in concanavalin A stimulated splenocytes of mice treated with Δ^9-THC (Massi *et al.*, 1998), PMA/Ionomycin-stimulated mouse splenocytes treated with 2-AG (Kaplan *et al.*, 2005a) and human T lymphocytes stimulated with allogeneic dendritic cells and Δ^9-THC (Yuan *et al.*, 2002). One study on human subjects showed that macrophages from marijuana smokers were compromised in their ability to produce TNFα, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-6 in response to LPS stimulation (Klein, 2005). LPS-induced TNFα release was also inhibited by AEA, 2-AG, WIN55,212-2, CP55,940 and HU210 in primary

cultures of rat cortical microglial cells (Facchinetti *et al.*, 2003) and in the macrophage cell line RAW264.7, the decrease in supernatant TNFα levels elicited by Δ^9-THC treatment was due to an inhibition of the conversion of the pro-peptide to the secreted protein (Klein *et al.*, 1998b). Furthermore, Δ^9-THC and AEA decreased levels of LPS-induced IL-1α and β and IL-6 mRNA expression in rat microglial cells (Puffenbarger *et al.*, 2000). Δ^9-THC also inhibited CCL3 and CCL4, CCL5, TNFα, GM-CSF and IFNγ production in a NK cell line stimulated with PMA, IL-10 in HUT-78 HTVL-1 positive T lymphocyte cell line and CCL4 in a HTVL-1 positive B lymphocyte cell line (Srivastava *et al.*, 1998).

However, cannabinoids have also been shown to increase the production of cytokines in certain studies. One early study showed that TNF and IL-6 were found to be increased in serum collected from mice which were treated with Δ^9-THC before and after infection with *Legionella pneumophila.* The second injection of Δ^9-THC led to acute collapse and death and this was attributed to the increased mobilisation of these acute phase cytokines (Klein *et al.*, 1998a). Another showed Δ^9-THC treatment of endotoxin stimulated cultured mouse peritoneal macrophages increases the amount of IL-1α and β bioactivity in supernatants compared to endotoxin treatment alone. Δ^9-THC was found to affect processing and release of IL-1 rather than increase protein production (Klein *et al.*, 1998a). More recently, other papers have also shown that these and other cytokines and chemokines can be upregulated by cannabinoids (Croxford & Yamamura, 2005, Klein, 2005). For instance, Δ^9-THC increased TGFβ production in human, anti-CD3 stimulated peripheral blood lymphocytes in a CB_2R-dependent manner (Gardner *et al.*, 2002) and CP55,940, a synthetic cannabinoid, was shown to increase CXCL8, CCL2, CCL4 and TNFα mRNA and protein production in wild type and CB_2R overexpressing HL-60 cells (Jbilo *et al.*, 1999, Derocq *et al.*, 2000). The effects on CXCL8 and CCL2 were shown to be CB_2R-dependent (Jbilo *et al.*, 1999).

Thus, whilst it is clear that cannabinoids can modulate cytokine and chemokine production it is not clear whether in this capacity they are pro or anti-inflammatory. They can enhance and suppress production of both pro and anti-

inflammatory cytokines in a cell-type and cannabinoid specific manner (Srivastava *et al.*, 1998). Furthermore, one study has shown that cannabinoid concentration can play a role in deciding whether cytokines are up or downregulated. In LPS-stimulated human PBMCs 3nM Δ^9-THC inhibited TNFα, IL-6 and CXCL8 release whilst 3μM stimulated their release (Berdyshev *et al.*, 1997). Whilst some of the responses seen have been attributed to specific cannabinoid receptors, some cannabinoid receptor-independent effects have also been described (Puffenbarger *et al.*, 2000, Facchinetti *et al.*, 2003, Kaplan *et al.*, 2005a, Klein, 2005). Thus, *in vivo*, many factors are likely to decide whether cannabinoids are pro or anti-inflammatory with respect to cytokine production.

1.3.2.2 Modulation of Th1/Th2 cytokine balance

Naïve $CD4^+$ T lymphocytes can differentiate into Th1 or Th2 cells depending on the local environment and thereby help regulate cell-mediated (Th1) and humoral (Th2) immune responses. A shift towards Th1 has been associated with disease progression whereas a shift towards Th2 can be associated with therapeutic benefits (Croxford & Yamamura, 2005). Initially it was shown that Δ^9-THC suppressed cell-mediated immunity to *Legionella pneumophila* infection when injected into mice before infection (Klein *et al.*, 2000). It also appeared to cause an increase in Th2 activity, shifting the normal Th1/Th2 balance to favour a Th2-mediated response. Further studies have gone on to show that Δ^9-THC suppresses the Th1 responses by inhibiting the production of IFNγ and IL-12, as well as expression of IL-12 receptor β2 mRNA, and biases it towards Th2 by increasing the production of IL-4 (Klein *et al.*, 2000). Both the CB_1R and CB_2R have been implicated in the Th biasing response in the mouse *Legionella* infection model (Klein *et al.*, 2000) with the CB_1R being important in the inhibition of IL-12 receptor expression (Klein *et al.*, 2004). Other laboratories have also reported similar biasing to a Th2-mediated response (Roth *et al.*, 2002, Klein *et al.*, 2003, Klein, 2005). For instance, Δ^9-THC inhibited IFNγ mRNA expression and increased IL-4 mRNA expression in human peripheral blood lymphocytes activated using allogeneic dendritic cells (Yuan *et al.*, 2002) and it depressed cell-mediated immunity and increased production of IL-10 in mice injected with tumour cells (Yuan *et al.*, 2002). These effects were CB_2R

mediated (Yuan *et al.*, 2002). In contrast, one study, in which mice were injected with 2-AG, a foreign soluble protein and toll-like receptor agonist during primary immunization, showed that 2-AG shifted the response to a Th1 phenotype via activation of CB_2R in dendritic cells (Maestroni, 2004).

1.3.2.3 Modulation of IL-2 production

Cannabinoids have been reported to both positively (Nakano *et al.*, 1993, Jan & Kaminski, 2001, Jan *et al.*, 2002) and negatively (Condie *et al.*, 1996, Massi *et al.*, 1998, Ouyang *et al.*, 1998, Herring *et al.*, 2001, Ihenetu *et al.*, 2003a, Rockwell & Kaminski, 2004) modulate IL-2 expression by T lymphocytes. The magnitude of T lymphocyte activation appears to be the principal factor that governs the differential regulation of IL-2 by one of the natural cannabinoids, cannabinol (Jan & Kaminski, 2001). In primary mouse splenocytes and EL4.IL-2 murine thymoma cells, it was shown that suboptimal activation of T lymphocytes in the presence of cannabinol resulted in a stimulation of IL-2 production, whilst maximal activation resulted in suppression of IL-2 production by cannabinol.

Currently relatively little is known about how cannabinoids modulate IL-2 production. Initially, inhibition of cAMP was thought to be involved in cannabinoid-induced negative modulation of IL-2 expression (Condie *et al.*, 1996) and there is evidence to suggest that suppression of cAMP can lead to suppression of IL-2 (Kaplan *et al.*, 2005b). However, several papers have suggested that inhibition of cAMP does not seem to be the primary mechanism involved in modulation of IL-2 by cannabinoids. Instead, inhibition of NFκB and NFAT DNA binding activity may be involved (Ouyang *et al.*, 1998, Herring & Kaminski, 1999, Kaplan *et al.*, 2005b). An attentuation of ERK signalling has been associated with cannabinoid-induced suppression of IL-2 production (Herring *et al.*, 2001) whilst stimulation of ERK has been associated with cannabinoid-induced stimulation of IL-2 production (Jan & Kaminski, 2001). The role of the cannabinoid receptors in the modulation of IL-2 production has not been extensively studied although several papers have suggested that the effects are cannabinoid receptor-independent (Jan *et al.*, 2002, Kaplan *et al.*,

2003, Rockwell & Kaminski, 2004). Interestingly, one study has shown that AEA-induced suppression of IL-2 production in primary mouse splenocytes may be mediated by one of the COX-2 metabolites of AEA activating PPAR-γ (Rockwell & Kaminski, 2004).

1.3.3 Modulation of cell number

Initial experiments in the 1970s examined the proliferative ability of peripheral blood lymphocytes isolated from marijuana smokers and some found that proliferation was inhibited whilst others saw no effect (Klein *et al.*, 1998a, Croxford & Yamamura, 2005). Since then no clear consensus on how cannabinoids affect cell number has been reached as they have been shown to both enhance and suppress immune cell numbers.

It has been demonstrated that in mice treated with Δ^9-THC for 14 days there is a 20% reduction in the number of T lymphocytes and a 26% rise in the number of B lymphocytes in the splenocyte population (Massi *et al.*, 1998). In contrast, it was found that after 24 hour treatment with Δ^9-THC there was a general decrease in splenocyte cell number rather than a specific effect on T or B lymphocytes (McKallip *et al.*, 2002b). Similarly, it was determined that in human subjects who ingested bhang for 6-36 months there was a reduction in the number of all the various immune subpopulations (EL-Gohary & Eid, 2004).

Studies have shown that, *in vitro*, splenocytes from mice treated with Δ^9-THC for two weeks have a reduced proliferative response to mitogens such as concanavalin A (Massi *et al.*, 1998, Steffens *et al.*, 2005b). This was also apparent when mice were treated with Δ^9-THC for only 6 hours (McKallip *et al.*, 2002b). Similarly, murine splenocytes treated with Δ^9-THC *in vitro* also showed decreased proliferative responses to mitogens (Pross *et al.*, 1990, Schatz *et al.*, 1993, McKallip *et al.*, 2002b). Δ^9-THC also suppressed human T lymphocyte proliferation stimulated by allogeneic dendritic cells (Yuan *et al.*, 2002), WIN55,212-2 inhibited the growth of Rec-1 mantle cell lymphoma cells in culture (Flygare *et al.*, 2005) and AEA inhibited mitogen-induced proliferation of human peripheral blood mononuclear cells (PBMCs; Schwarz *et al.*, 1994).

Apoptosis has been suggested to account for at least some of the cannabinoid-induced decrease in cell number detected in some studies (Schwarz *et al.*, 1994, Flygare *et al.*, 2005). Δ^9-THC and AEA have been demonstrated to induce apoptosis in several immune cell lines including Jurkat (McKallip *et al.*, 2002a, Sarker & Maruyama, 2003, Herrera *et al.*, 2005, Lombard *et al.*, 2005), MOLT-4 (McKallip *et al.*, 2002a), HL-60 (Sarker & Maruyama, 2003, Powles *et al.*, 2005), CEM (Powles *et al.*, 2005) and U937 human lymphoma cells (Maccarrone *et al.*, 2000). Δ^9-THC has also been shown to induce apoptosis of murine bone marrow derived dendritic cells (Do *et al.*, 2004), murine peritoneal macrophages and splenocytes (Zhu *et al.*, 1998), primary human acute lymphoblastic leukaemia cells (McKallip *et al.*, 2002a) and human PBMCs (Powles *et al.*, 2005).

Not many studies have looked at the mechanisms of cannabinoid-induced apoptosis in immune cells compared to other cell types. One noteworthy aspect is that generally high concentrations are required to induce apoptosis (Schwarz *et al.*, 1994, McKallip *et al.*, 2002a, Powles *et al.*, 2005). Both cannabinoid receptor-dependent (McKallip *et al.*, 2002a, Do *et al.*, 2004, Herrera *et al.*, 2005) and -independent apoptosis has been described (Sarker & Maruyama, 2003, Powles *et al.*, 2005). The VR1 has also been implicated in AEA-induced apoptosis of human lymphoma U937 cells (Maccarrone *et al.*, 2000) and a role for lipid rafts in AEA-induced apoptosis of Jurkats and HL-60 cells was also demonstrated (Sarker & Maruyama, 2003). The MAPK p38 was shown to play a role in Δ^9-THC induced apoptosis of Jurkats (Herrera *et al.*, 2005). Activation of NFκB and caspase-2, -8, -9 and -10, cleavage of Bid, decrease in Bcl-2, decreased mitochondrial membrane potential and cytochrome *c* release have also been implicated in cannabinoid-induced apoptosis of immune cells (Zhu *et al.*, 1998, Do *et al.*, 2004, Lombard *et al.*, 2005).

Cannabinoids have also been reported to increase proliferation of immune cells. Δ^9-THC, CP55,940 and WIN55,212-2 increased proliferation of activated human B lymphocytes in a PTX-sensitive manner. It was suggested that the CB_2R was involved in this response (Derocq *et al.*, 1995). 2-AG, but not AEA, was also

shown to enhance proliferation of a M-CSF-dependent rat microglial cell line in the presence of M-CSF. This response was mimicked by the CB_2R-selective agonist JWH-133 and blocked by the CB_2R antagonist SR144528 (Carrier *et al.*, 2004). However, AEA did enhance growth factor-dependent proliferation of a number of haematopoietic growth factor-dependent murine cell lines (Valk *et al.*, 1997) and cytokine-dependent proliferation of the murine IL-6-dependent lymphoid cell line B9 and IL-3-dependent myeloblastic cell line FDC-P1 (Derocq *et al.*, 1998). In the latter study the AEA effect was shown to be cannabinoid receptor-independent (Derocq *et al.*, 1998). In all of these studies the cannabinoids on their own had no effect on proliferation, their effect was on stimulated proliferation.

It has been suggested that cannabinoid effects on lymphocyte proliferation may be concentration-dependent (Luo *et al.*, 1992). Δ^9-THC increased PHA or concanavalin A-stimulated proliferation of human peripheral blood lymphocytes and murine splencoytes at low concentrations but suppressed proliferation at high concentrations (Luo *et al.*, 1992). Equally, whilst nanomolar concentrations of CP55,940 enhanced proliferation of activated human B lymphocytes, high micromolar concentrations inhibited proliferation (Derocq *et al.*, 1995). Thus, the effects of cannabinoids on immune cell number may be concentration-dependent in certain circumstances and studies using high concentrations may need to be reviewed with this data in mind.

1.3.4 Antibody production

Several early reports have shown that cannabinoids suppress serum immunoglobulin (Ig) levels and antibody formation. Several *in vivo* and *in vitro* studies have demonstrated that cannabinoids generally inhibit antibody formation. In humans and primates it has been shown that either marijuana smoking or ingestion of bhang decreases serum levels of IgG although the effects on other Ig types was more variable (Klein *et al.*, 1998a, EL-Gohary & Eid, 2004, Croxford & Yamamura, 2005). The duration of exposure and dose affected the extent to which IgG levels were decreased. Studies in mice, both *in vivo* and *in vitro,* using isolated splenocytes have also shown that Δ^9-THC

suppresses the antibody forming response (Klein *et al.*, 1998a). As antibody formation requires co-ordinated interaction between T lymphocytes, macrophages and B lymphocytes for maximal response, suppression of any or all of these cell types might reduce the type and amount of antibody produced. Although it is still not clear exactly what mechanisms are involved in the cannabinoid suppression of antibody production, one study has suggested that it is T lymphocyte-dependent (Schatz *et al.*, 1993). Δ^9-THC administered to mice or to murine splenocytes suppressed the antibody response to sheep red blood cells which is a T lymphocyte-dependent response, but not to DNP-Ficoll which is a T lymphocyte-independent response (Schatz *et al.*, 1993). Another study also implicated cannabinoid suppression of cAMP signalling in the inhibition of antibody production (Klein *et al.*, 1998a, Berdyshev, 2000).

1.4 Aims of the Project

The initial aim of the study was to further work carried out in the colonic epithelial cell line, HT-29, that showed ERK1/2 activation downstream of cannabinoids. This was to be done by first repeating data already collected within the group and extending it to additional cell lines and other signalling pathways in order to develop our understanding of cannabinoid receptor signalling in colonic epithelial cells. This idea stemmed from work being done in the group which showed that whilst only the CB_1R was present on normal colonic epithelial cells, the CB_2R could be detected in the epithelium of colonic sections taken from patients with inflammatory bowel disease (IBD) indicating an immunomodulatory role for the cannabinoids in mucosal tissue (Wright *et al.*, 2005). However, problems were encountered in that the cell lines used expressed moderate to high basal levels of phosphorylated ERK1/2 and these were significantly augmented when cells were treated with vehicle, making data with the cannabinoids both inconsistent and difficult to interpret.

Therefore, the focus of the project shifted towards the effects of cannabinoid on T lymphocyte function. T lymphocytes were chosen as they play an important role in inflammatory diseases such as IBD (MacDonald & Monteleone, 2005) and although the effects of cannabinoids on T lymphocytes have begun to be

explored much of the data available is contradictory and studies have not always used human cells.

Thus the aims of the project were as follows:

- To investigate the expression of cannabinoid receptor protein in activated human T lymphocytes and determine whether this differs from naïve T lymphocytes.
- To determine whether cannabinoids couple to intracellular signalling pathways in activated T lymphocytes and whether the effects detected are receptor mediated or not.
- To explore the effects of cannabinoids on migration of activated T lymphocytes.
- To explore the effects of cannabinoids on proliferation of activated T lymphocytes.

Chapter 2: Materials & Methods

2.1 Materials

2.1.1 Cell culture materials

Cell culture media, fœtal bovine serum, penicillin and streptomycin, trypan blue, trypsin-EDTA and PBS (without Ca^{2+} and Mg^{2+}) were purchased from Gibco® (Invitrogen, Paisley, UK). Lymphoprep (Ficoll-paque 1.077g/ml density) was purchased from Axis-Shield (Oslo, Norway). Staphylococcal enterotoxin B was purchased from Sigma-Aldrich (Gillingham, UK) and Dynabeads® CD3/CD28 T cell expander beads from Dynal Biotech (UK). IL-2 was purchased from Chemicon (Hampshire, UK). All plastics were obtained from Nunc (UK), except cryotubes which were purchased from Fisher Scientific (Loughborough, UK).

2.1.2 Antibodies

2.1.2.1 For immunoblotting

Rabbit polyclonal anti-CB_1R antibodies were purchased from Affinity Bioreagents (Cambridge Bioscience, Cambridge, UK ; catalogue no. PA-743), Cayman Chemicals (Michigan, USA ; catalogue no. 101500) and Santa Cruz Biotechnologies (California, USA ; catalogue no. sc-20754). Rabbit polyclonal anti-CB_2R antibodies were obtained from Cayman Chemicals (Michigan, USA ; catalogue no. 101550) and Santa Cruz Biotechnologies (California, USA ; catalogue no. sc-25494). CB_1R and CB_2R blocking peptides were also purchased from Cayman Chemicals (Michigan, USA). A rabbit polyclonal anti-FAAH antibody was purchased from Alpha Diagnostics International (Texas, USA ; catalogue no. 11-A) and a rabbit polyclonal anti-MAGL antibody was obtained from Cayman Chemicals (Michigan, USA ; catalogue no. 100035). Rabbit polyclonal anti-phospho $ERK1/2^{Thr202/Tyr204}$ (catalogue no. 9101) and anti-phospho PKB^{Ser473} (catalogue no. 9271) were purchased from Cell Signalling Technologies (UK). Rabbit polyclonal pan ERK (catalogue no. sc-93) and anti-IκBα (catalogue no. sc-371) and goat polyclonal pan PKB (catalogue no. sc-1618) antibodies were obtained from Santa Cruz Biotechnologies (California,

USA). HRP conjugated anti-goat and anti-rabbit immunoglobin were purchased from Dako (Glostrup, Denmark).

2.1.2.2 For flow cytometry

A mouse monoclonal anti-CD3 (clone UCHT1) antibody was obtained from Doreen Cantrell (School of Life Sciences, Dundee, UK) and a mouse monoclonal anti-CD28 (clone 9.3) was obtained from Carl June (University of Pennsylvania, USA). Mouse monoclonal R-PE-conjugated anti-human CD3 (catalogue no. 555340), FITC-conjugated anti-human CD8 (catalogue no. 555366) and R-PE-conjugated IgG1 and IgG2 were purchased from BD Biosciences (Oxford, UK).

2.1.3 Other

2-AG, ACPA, AEA, AM251, AM630, JWH-133, MAFP, (R)-(+)-methanandamide, and LY294002 were purchased from Tocris Cookson (Avonmouth, UK). Gö6976, PD98059, Ro-32-0432, rottlerin, PMA and tunicamycin (source *Streptomyces lysosuperficus*) were purchased from Calbiochem (Nottingham, UK). FITC-conjugated phalloidin was obtained from Alexis Biochemical (Axxora Ltd, Nottingham, UK). Acrylamide/bis acrylamide and molecular weight protein markers were purchased from Bio-rad (UK). Versene was purchased from Gibco® (Invitrogen, Paisley, UK). Bromophenol blue, nitrocellulose and hydrochloric acid were obtained from BDH (Poole, UK). Cell Titer 96® Aqueous One Solution Cell Proliferation Assay was purchased from Promega UK (Southampton, UK). The Cyclic AMP (low pH) Immunoassay and recombinant human CXCL12 were obtained from R&D Systems (Abingdon, UK). The pan T cell isolation kit II was purchased from Miltenyi Biotec (California, USA). The Enhanced chemiluminescence detection kit and X-OMAT film were purchased from Amersham Internationl (UK). Ethanol, methanol and isoton II were obtained from Fisher Scientific (Loughborough, UK). The filter paper used in immunoblotting was purchased from Whatman (Maidstone, UK). Marvel was purchased from the local supermarket. All general plastic ware was obtained from Greiner-Bioone (Germany), including the 96-well plates used in the *in vitro* cell migration assay.

All other reagents, including pertussis toxin (source *Bordella pertussis*) were purchased from Sigma-Aldrich (Gillingham, UK).

2.2 Cell types and culture conditions

2.2.1 General

All cells were routinely maintained in their respective media supplemented with penicillin (100U/ml), streptomycin (100μg/ml) and 10% (v/v) foetal bovine serum (FBS, referred to as complete media). The medium was changed every 2 to 3 days and cultures were maintained at 37°C in an atmosphere of 5% CO_2. Prior to each experiment cells were counted and cell viability checked by trypan blue exclusion. 40μl of cell suspension was mixed with 40μl of trypan blue and cells counted using a Neubauer haemocytometer. Cell viability was always greater than 95%.

2.2.2 Jurkat cells

The human leukaemic T lymphocyte cell line Jurkat was obtained from the Imperial Cancer Research Fund. Jurkats were cultured in complete RPMI 1640 medium in 175cm^2 tissue culture flasks and split into additional flasks when confluent.

2.2.3 CEM cells

The human Caucasian acute T lymphoblastoid leukaemia cell line CEM was obtained from Dr. Z. Brown (Novartis, Horsham, UK). CEMs were cultured in complete RPMI 1640 medium in 175cm^2 tissue culture flasks and split into additional flasks when confluent.

2.2.4 HT-29 cells

The human colonic epithelial cell line HT-29 was obtained from the European Collection of Animal Cell Cultures. HT-29 cells are human colon

adenocarcinoma cells isolated from a primary tumour in a 44 year old Caucasian female. HT-29s were cultured as monolayers in complete McCoy's 5A medium in 80cm^2 tissue culture flasks. Confluent monolayers were subcultured as follows. The medium was removed from the flask and the monolayer washed twice with phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}) before being washed with 3ml of a Trypsin-EDTA (0.05% (w/v) Trypsin and 0.02% (w/v) EDTA). The excess Trypsin-EDTA was removed and cells incubated at 37°C in an atmosphere of 5% CO_2 for up to 5 minutes to allow the HT-29s to detach from the tissue culture flask. The action of the Trypsin-EDTA was stopped by adding approximately 20ml of complete medium. The cells were spun down at 1,500rpm for 5 minutes and the media removed to wash out the Trypsin-EDTA. The pellet was resuspended in fresh complete media before being counted and viability checked as described above. Cells were then seeded into a tissue culture flask and/or into a variety of dishes as required for experiments.

2.2.5 Activated peripheral blood-derived T lymphocytes (PBLs)

Activated human PBLs were generated from PBMCs as described below and then maintained in complete RPMI 1640 medium supplemented with 50U/ml of IL-2 in 80cm^2 or 175cm^2 tissue culture flasks. When confluent, these were split into additional or larger flasks.

2.2.6 Freezing and thawing of cells

The cell lines were stored frozen in liquid nitrogen tanks to provide a stock of cells of a particular age and to minimise the transformation of cell lines that may occur with long-term cell culture. Growing cells were resuspended at 2-10x10^{6}cells/ml in 'freeze media' (90% (v/v) FBS, 10% (v/v) dimethylsulphoxide (DMSO)) and quickly transferred to cryotubes at 1ml/tube. These tubes were then slowly frozen at a rate of 1°C/min to reduce to the occurrence of artefacts prior to being stored in liquid nitrogen tanks. When cells were thawed the cryotube was held in a 37°C water bath to rapidly defrost the cell suspension. Once thawed the cells were quickly resuspended in their respective complete media and spun down at 1,500rpm for 5 minutes. The cells were resuspended in

fresh complete media, seeded into a 80cm^2 tissue culture flask and maintained as described above.

2.3 Generation of activated PBLs *in vitro*

2.3.1 Overview

In this study, activated human PBLs were cultured in order to investigate the expression and role of the cannabinoid receptors in activated T lymphocytes. These cells are T lymphocytes that have been activated *in vitro* and clonally expanded using IL-2. Briefly, PBMCs are isolated as described below and the T lymphocytes present in the population activated using the superantigen Staphylococcal enterotoxin B (SEB) or CD3/CD28-coated beads. SEB acts by binding to the class II MHC molecules expressed on professional APCs, such as the B lymphocytes found in the PBMC population, and then sequentially binding the TCR, bringing them together to induce T lymphocyte activation (Proft & Fraser, 2003). In contrast, the CD3/CD28-coated beads mimic the stimulation of T lymphocytes by APCs by binding both CD3 and CD28.

2.3.2 Isolation of PBMCs by density gradient centrifugation

Blood (50-100ml) was collected from healthy volunteers in heparinised syringes (500U/50ml syringe) and diluted 1:1 with RPMI 1640 medium. 35ml of the blood/RPMI mix was then carefully layered over 15ml of lymphoprep in a 50ml falcon tube before being centrifuged at 1,500rpm at room temperature for 30 minutes with the brake off. After centrifugation, the milky layer of PBMCs, shown in Fig. 2.1, containing the lymphocytes and monocytes was swiftly removed into a fresh tube. This fraction was diluted 1:1 with RPMI 1640 and the cells washed 3 times to remove any lymphoprep collected during the isolation. After the final wash the cells were resuspended in complete RPMI 1640 at a density of 1x10^{6}cells/ml and then set up as described below.

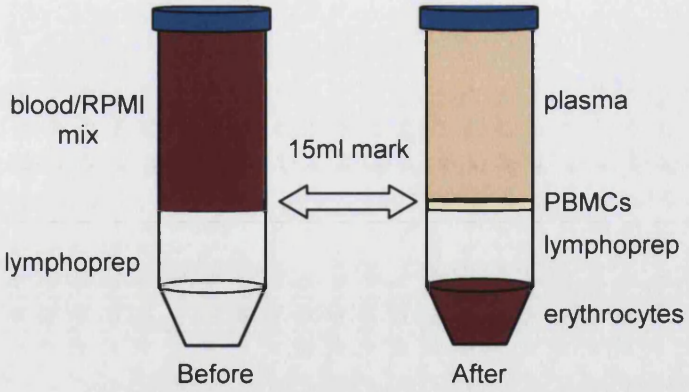


Figure 2.1: **Schematic showing the falcon tube containing blood and lymphoprep before and after centrifugation.**

2.3.3 Activation of T lymphocytes using SEB

The PBMCs were placed in a 175cm^2 tissue culture flask and 1µg/ml of SEB was added to activate the T lymphocytes. After 72 hours the cells were washed three times in RPMI 1640 and then placed into a new 175cm^2 tissue culture flask at a density of 1x10^{6}cells/ml in complete RPMI 1640 and IL-2 added as described above to clonally expand the activated T lymphocytes. These activated PBLs were thus further maintained until they were set up for experiments. Cells were cultured for a total of 2 weeks.

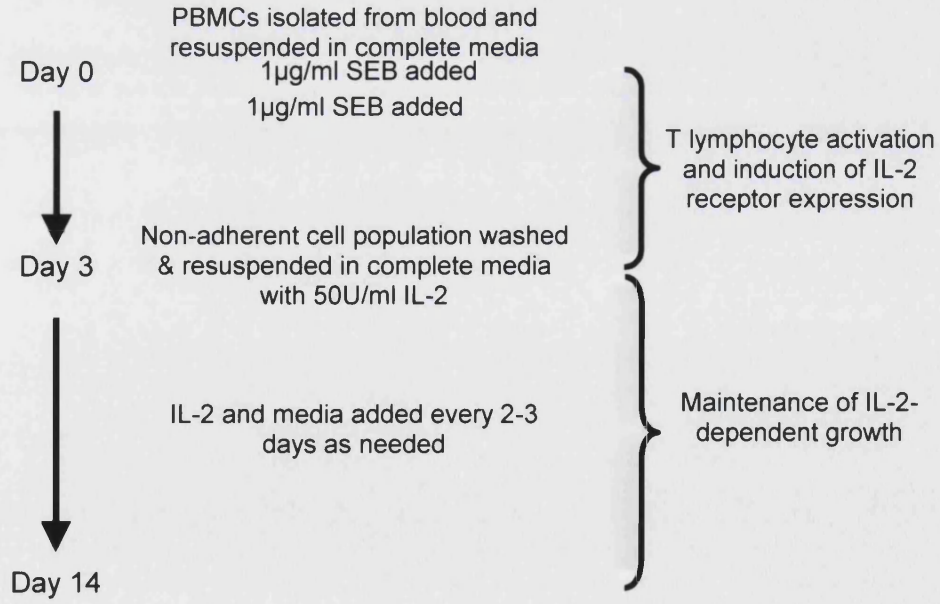


Figure 2.2: **A summary of the preparation of SEB-activated PBLs.**

2.3.4 Activation of T lymphocytes using CD3/CD28-coated beads

Approximately $2x10^7$ cells in complete RPMI 1640 were placed in a $80cm^2$ tissue culture flask and incubated for up to 3 hours to allow the monocytes to adhere to the plastic. The remaining cells were then counted as described above and resuspended in complete RPMI 1640 at $1x10^6$cells/ml. Dynal® CD3/CD28 T cell expander beads were then added at 2 beads/cell and the complete media supplemented with 50U/ml of IL-2. Subsequently these activated PBLs were maintained as described above under the cell culture conditions for a total of 3 weeks.

2.4 Purification of naïve human peripheral T lymphocytes

2.4.1 Isolation of naïve T lymphocytes

Naïve human T lymphocytes were purified from freshly isolated PBMCs using a human Pan T Cell Isolation Kit (Miltenyi Biotec). The system works by indirectly magnetically labelling all of the non-T lymphocytes in the PBMC population by using a cocktail of biotin-labelled monoclonal antibodies (against CD14, CD16, CD19, CD36, CD56, CD124 and glycophorin A) and then adding in anti-biotin microbeads. By retaining the magnetically labelled cells in a column, a pure population of T lymphocytes can be isolated.

The protocol was followed as per the manufacturers instructions. Briefly, PBMCs were isolated from human blood as described above being especially careful to avoid collecting any erythrocytes and lymphoprep as far as possible as these will affect the purity of the population. Cells were resuspended in PBS (without Ca^{2+} and Mg^{2+}) containing with 2mM EDTA and 0.5% bovine serum albumin (BSA; referred to as buffer A) and centrifuged at 1,500rpm for 15 minutes in a 50ml falcon tube. The buffer was removed and the pellet resuspended in 10ml of buffer A and transferred to a 15ml falcon tube. The cells were washed twice in buffer A and as many platelets removed as possible. The remaining cells were resuspended in buffer A at a density of 40µl of bufferA/$1x10^7$ cells. To this, 10µl of Pan T cell Biotin-Antibody Cocktail (human) was added per $1x10^7$ cells. This was left to incubate on ice for 15

minutes. The cells were then washed to remove excess unbound antibody and resuspended in buffer A at a density of 30µl bufferA/1x10^7 cells. 20µl of Anti-Biotin MicroBeads/1x10^7 cells were then added and the cells again incubated on ice for 15 minutes. After this the cells were washed in buffer A. During this time the MACS column was prepared by slowly running 3ml of buffer A through the column. The cells were resuspended in 500µl of buffer A and run through the column. The cells that have bound antibody bind to the column, so the T lymphocytes could be collected as they ran through. 1ml of buffer A was subsequently run through the column in order to ensure that as many T lymphocytes were collected as possible. At this stage cells were removed from the population to be evaluated for purity and kept on ice in buffer A. The remaining cells were spun down and stored at –80°C for later use or used immediately in stimulations as described below.

2.4.2 Evaluation of T lymphocyte population purity

In order to verify that the purification procedure was successful flow cytometry analysis of the percentage of cells expressing CD3 and CD28 was carried out as described later on. Briefly, the cells were analysed for autofluorescence, the expression of CD3 using an anti-CD3 antibody (clone UCHT1, 1µg/ml) and the expression of CD28 using and anti-CD28 antibody (clone 9.3, 1µg/ml). An isotype control (mouse IgG) was also set up. Purity was always >93%.

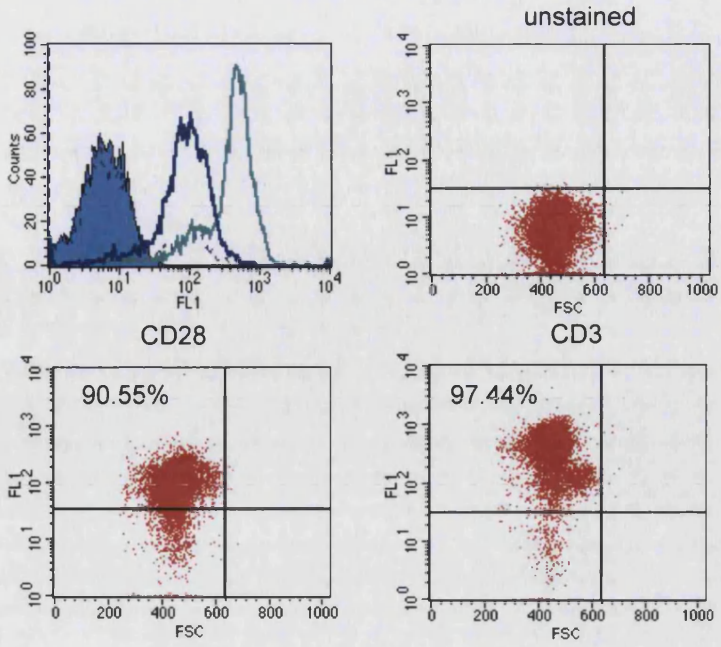


Figure 2.3: **Evaluation of T lymphocyte population purity.** Naïve T lymphocytes were purifed from human PBMCs as described above and flow cytometry was used to determine the percentage purity of the population isolated. The expression of CD3 and CD28 was verified by staining the cells with anti-CD28 antibody (— dark blue line), anti-CD3 antibody (— green line) and isotype control (— purple line) followed by flow cytometric analysis. Some cells were also left unstained (light blue histogram) to ascertain autofluorescence. Purity was always >93%. Data are representative of three separate experiments.

2.5 Stimulations in suspension cells

The day prior to the experiment, activated PBLs were washed three times in RPMI 1640 to remove IL-2 and resuspended in complete media overnight. On the day of the experiment, all suspension cells were washed three times in RPMI 1640 and counted as described above. They were then resuspended at a density of 2-10x10^6cell/ml and 500µl of cell suspension used per stimulation. Cells were placed in 1.5ml plastic tubes and allowed to rest in a 37°C water bath for 1 hour. During this hour any pre-treatments necessary were added to the cells. To start the stimulation the appropriate cannabinoid, vehicle or other ligand was added to the cells and the cells returned to the water bath. To terminate the stimulation and generate whole cell lysates (WCLs) the cells were rapidly centrifuged at 10,000rpm, the media removed, ice cold lysis buffer added to solubilise the cells and the tube vortexed. 100µl of lysis buffer was used per 1x10^6 Jurkats and 2x10^6 activated PBLs or naïve T lymphocytes. The samples were kept on ice until all stimulations were completed. Once they were all completed the samples

were rotated for 10 minutes at 4°C and then centrifuged at 14,000rpm for 10 minutes at 4°C to remove any insoluble material. The protein containing supernatant was transferred to fresh 1.5ml plastic tubes. These WCLs could be frozen and stored at -80°C at this point.

2.6 Immunoblotting

2.6.1 Overview

Immunoblotting or Western blotting is a technique that allows visualisation of the relative amounts of specific proteins within cells. The cells are lysed and the whole proteins are extracted. These WCLs are then run in a current through a polyacrylamide gel to separate the proteins according to their molecular weight. The now separated proteins are then transferred to a membrane which can be probed for specific proteins using antibodies. Once this primary antibody has bound the protein of interest a secondary antibody, which has a horseradish peroxidase tag, is applied. The horseradish peroxidase tag on the secondary antibody catalyses a reaction which can then be visualised and represents a way of detecting the protein.

The primary antibodies that are used can be specific to a certain conformation of the protein of interest. For instance, they can be generated to bind the phosphorylated form of a protein. This type of primary antibody can be used to assess relative changes in amounts of phosphorylated protein, which may occur, for example, when the cells are stimulated by different ligands. In order to verify that the total amount of protein is unchanged, results from these phosphorylation site antibodies are compared to results using an antibody against all forms of the protein, usually referred to as a pan antibody.

2.6.2 Generating samples

2.6.2.1 General

Once lysates were generated and transferred to clean tubes, 5x sample buffer (see recipe in *Appendices*) was added and the samples boiled for 5 minutes. The sodium dodecyl sulphate (SDS) in the buffer acts to denature the proteins and the 2-mercaptoethanol reduces the disulphide bridges. In addition, the SDS binds to the polypeptides and gives them a negative charge in such a way that all of the SDS-polypeptide complexes have essentially the same negative charge and shape such that they migrate through the gel according to molecular size only.

2.6.2.2 Stimulated cells

Cells were stimulated as described above and WCL samples prepared by adding 5x sample buffer. Samples were always prepared such that the they represented 8,000 cells/μl sample for T lymphocytes cell lines and 16,000 cells/μl sample for activated PBLs and naïve T lymphocytes.

2.6.2.3 Unstimulated cells

In order to look at certain parameters, for instance receptor expression during the course of PBL culture, WCLs of cells on different days of culture were generated. In order to do this, 1-2x10^6 cells were removed from culture, spun down, the medium removed and the cells placed in a –80°C freezer for storage. In the case of the CD3/CD28-activated PBLs, care was taken to first remove the beads. Once the time course was completed all the samples were lysed in lysis buffer to generate the WCLs. Freshly isolated and purified T lymphocytes were also lysed to generate WCLs. The lysates were transferred to clean 1.5ml plastic tubes and a protein assay completed as described below and then 5x sample buffer added and the samples boiled. In this instance, each sample contained a different concentration of protein and different volumes of each sample were loaded onto gels in order to ensure equal loading.

Again, in order to verify receptor expression in the various cell lines used in this study, samples were also taken of these at intervals throughout culture and lysed to generate WCLs. For the HT-29s these were seeded into 6cm dishes at $0.8x10^6$ cells/dish and allowed to grow to approximately 80% confluency, before being solubilised by adding lysis buffer and using a cell scraper. For CEMs and Jurkats, approximately $5x10^6$ cells were removed from culture, spun down and the medium removed. The cells were then solubilised in lysis buffer to generate WCLs. Samples were transferred to clean plastic tubes and a protein assay carried out as described below. In this instance, each sample contained a different concentration of protein and different volumes of each sample were loaded onto gels in order to ensure equal loading.

2.6.2.4 Subcellular fractionations

In order to determine where in the cells certain proteins were located subcellular fractions were carried out to separate the cytosolic and membranous proteins. Alongside this protocol WCLs of each cell type were also prepared as described above as a control. Jurkats were washed three times in RPMI 1640 and then resuspended in ice cold hypotonic lysis buffer (see recipe in *Appendices*) at a density of $2x10^7$ cells/ml. HT-29s were seeded onto 10cm dishes 2-3 days in advance and grown to approximately 80% confluency. They were then solubilised by adding hypotonic lysis buffer and a cell scraper used to remove the cells from the dish.

The cells were left in the hypotonic lysis buffer on ice for 10 minutes to swell before being disrupted by sonication. The cells were disrupted using 3 x 2 second pulses at 20 second intervals and kept on ice. Before continuing, it was ensured that at least 80% of the cells were disrupted by looking at them under the microscope. The nuclei and any remaining intact cells were removed by centrifuging the cells at 13,000rpm at 4°C for 15-30 seconds. The supernatant was then centrifuged at 39,000rpm for 20 minutes at 4°C. The supernatant, which is the cytosolic fraction, was carefully removed, placed in clean tubes and kept on ice. The pellet was carefully rinsed in 500µl of hypotonic lysis buffer to ensure that all of the cytosolic fraction was removed so it would not contaminate

the membranous fraction. The pellet was then solubilised in 150µl of hypotonic lysis buffer containing 1% (v/v) Nonidet P-40, centrifuged at 13,000rpm for 5 minutes at 4°C to remove any remaining insoluble material and transferred to clean 1.5ml plastic tubes. Samples could be stored at –80°C at this point. Before 5x sample buffer was added a protein assay was carried out as described below such that they could be equally loaded onto SDS-PAGE gels according to protein content rather than on an equivalent cell basis.

2.6.2.5 Immunoprecipitations (IPs)

HT-29s were grown on 10cm dishes until they reached approximately 80% confluency. They were then solubilised by adding 1ml of lysis buffer and using a cell scraper. The samples were then centrifuged at 14,000rpm for 10 minutes at 4°C to remove any insoluble material and the supernatants were transferred to clean plastic tubes. Protein G sepharose beads (20µl of a 50% slurry with lysis buffer) was added to each sample and the samples rotated at 4°C for 30 minutes to preclear the samples and minimise non-specific binding. The beads were then removed from the samples and 5µl of antibody was added to each sample and the samples rotated at 4°C for 1 hour. 30µl of protein G sepharose beads (50% slury with lysis buffer) were then added to each sample and the sample rotated for another hour at 4°C. The samples were then centrifuged at 13,000rpm for 1 minute at 4°C and the supernatant removed. Fresh lysis buffer was added to wash the beads which now had the protein of interest attached to them. This process was repeated 3 times. After the final wash the supernatant was carefully removed and 15µl of 2x sample buffer was added (5x sample buffer diluted with MilliQ). The samples were boiled for 5 minutes as for other samples. The entire sample was loaded onto gels. Alongside the IPs, WCLs of unstimulated HT-29s were also generated as described above to run as comparison.

2.6.3 Protein assay

In certain cases a protein assay was carried out in order to determine the concentrations of protein within samples generated such that equal quantities could be loaded onto SDS-PAGE gels. Total protein per lysate was estimated

using the Bio-Rad Protein assay. Bradford solution was diluted in MilliQ water to 20%. For each sample, 5µl of cell lysate was added to 1ml of diluted Bradford reagent and vortexed. 100µl of this Bradford sample was then transferred to a 96 well plate in duplicate. A standard curve was also generated using known concentrations of BSA and added to the plate. The plate was then read at 595nm on a VersaMax platereader. The protein concentrations were calculated by linear regression from the standard curve.

2.6.4 Separation of cellular proteins by electrophoresis

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecule size. Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out using the Bio-Rad Mini Protean II system (Biorad Labs, Hemel Hemptstead, UK).

Minigels were prepared following the recipes described in the appendix (10% resolving gels were prepared unless otherwise stated). The resolving gel was poured into the gel equipment and overlaid with MilliQ water to create a uniform surface. After approximately 30 minutes, once the gel had set, the water was removed, the stacking gel poured on top and combs inserted to create wells. After another 10 minutes, once the stacking gel had set, the combs were removed and the wells were thoroughly cleaned with MilliQ water before being filled with running buffer (see recipe in *Appendices*). The samples were then loaded, typically 30µg of protein or the equivalent of $1.6x10^6$ Jurkats or $3.2x10^6$ activated PBLs. In one of the wells 5µl molecular weight markers were loaded for ease of protein band identification later on. The gels were then run at room temperature at 80 volts until the bromophenol blue tracking dye in the samples entered the resolving gel. The voltage was then increased up to 180 volts. Gels were run until the bromophenol blue dye reached the bottom of the resolving gel. Gels were then placed into semi-dry transfer buffer (see recipe in *Appendices*).

2.6.5 Semi-dry transfer of proteins to nitrocellulose

Once the proteins were separated according to size they were transferred onto nitrocellulose membrane. Firstly the graphite electrodes of the transfer apparatus (Pharmacia-Biotech Multiphor II) were dampened with semi-dry transfer buffer. Four pieces of 3MM Whatman paper, cut to the same size as the gel, soaked in semi-dry transfer buffer were placed on the bottom electrode and rolled to gently expel any airbubbles. On top of this a piece of nitrocellulose membrane, again cut to be the same size as the gel, also soaked in semi-dry transfer buffer, was placed. The gel was then placed on top of this before another 4 wet pieces of 3MM Whatman paper were placed on top. Once this 'sandwhich' was assembled it was again gently rolled to expel airbubbles. The transfer was run for 1 hour at 0.8mA/cm^2 of membrane (approximately 40mA per gel). Once the transfer was finished the membrane was stained using Ponceau S to verify whether the transfer had occurred evenly. The stain was removed by briefly washing the membranes in MilliQ water before washing them for up to 10 minutes in Tris-buffered saline (TBS, see recipe in *Appendices*).

2.6.6 Immunoblotting of nitrocellulose bound protein

Once the Ponceau S stain was completely removed, non-specific binding sites on the nitrocellulose were blocked by incubating the membranes with the appropriate blocking buffer (see conditions for each antibody in the *Appendices*) for 1 hour at room temperature. After this time the blocking buffer was removed and the membrane washed briefly in TBS-Tween (TBST, see recipe in *Appendices*) to remove excess blocking buffer. The membranes were then incubated in primary antibody, which was diluted in TBS (as stated in the *Appendices*), overnight at room temperature. After this the primary antibody was removed and the membranes were then washed 3 times for 10 minutes in TBST to remove any excess antibody before the secondary antibody (diluted in TBST as mentioned in the *Appendices*) and was added to the membranes. The secondary antibody was left on the membranes for 1-3 hours and the membranes then again washed 3 times for 10 minutes in TBST. The membranes were then finally washed for 5 minutes in TBS. 5ml of Enhanced Chemiluminescent

Lumigen reagent was added for 1 minute and the membranes were then exposed to X-ray film for 2 seconds – 2 hours and the film developed using an RGII Fuji X-ray film developer. During this incubation periods and the washes the membranes were rocked on a moving platform to ensure even distribution of the blocking buffer and antibody or to ensure even washing of the membrane surface.

2.6.7 Membrane stripping and reprobing

In order to verify whether the samples were equally loaded the nitrocellulose membranes were stripped free of antibody and reprobed with a pan antibody. Firstly, membranes were rehydrated in TBS. Each membrane was then placed in a sealed container with approximately 25ml of stripping buffer (see recipe in *Appendices*), and the container placed in a water bath, which was preheated to 60°C, for 30 minutes. During this time the container was frequently shaken to ensure the blot was properly stripped. After the half hour the stripping buffer was disposed of, the blots moved to clean containers and washed three times in TBST for 10 minutes each time. After a final fourth wash in TBS the membranes were then again blocked as before and a new pan primary antibody placed on them, also as before. The rest of the procedure is identical to normal immunoblotting.

2.6.8 Densitometric analysis

Densitometric analysis was carried on some blots. Optical densitometry was measured using the GeneTools software from SynGene and values were expressed as fold change over vehicle.

2.7 *In vitro* cell migration assay

An assay based on the Boyden Chamber system was used to examine cell migration *in vitro*. In this assay a 96-well plate is set up with appropriate ligands and placed in the bottom of the chamber. A filter is then applied through which the cells will migrate. The chamber is then closed and in the top wells, which

form part of the chamber structure and coincide with the bottom 96-well plate, cells are placed and they are allowed to migrate.

Specifically, a 96-well chemotaxis chamber from NeuroProbe (Fig. 2.4; Gaithersburg, MD) was used. The day prior to the experiment, activated PBLs were washed three times in RPMI 1640 medium to remove IL-2 and resuspended in complete media overnight. On the day of the experiment, all suspension cells were washed three times in RPMI 1640 and counted as described above. They were then resuspended at a density of $1x10^6$cell/ml in RPMI 1640 supplemented with 0.1% BSA and allowed to rest in a 37°C water bath for 1 hour, during which time any appropriate pre-incubations were carried out. A clear bottom 96-well plate was set up with 395µl of agonist or vehicle controls, diluted to the appropriate concentration in RPMI 1640 supplemented with 0.1% BSA, per well. This was then placed in the lower part of the chamber. A polyvinylpyrrolidine-free polycarbonate filter (5µm pore size) was carefully placed on top. The chamber was closed and 200µl of cell suspension added to each well of the upper chamber. A minimum of triplicates was set up for each condition tested. The chamber was then incubated for 3 hours if activated PBLs were being used or 90 minutes for Jurkats and CEMs in at 37°C in an atmosphere of 5% CO_2. After this time the suspension from the top wells of the chamber was removed and 200µl of versene placed in each well to dissociate cells adhering to the filter for 20 minutes. The versene was removed and the filter covered 96-well plate was centrifuged at 1,500rpm for 10 minutes. The media was then carefully removed and the cells in each well resuspended in 100µl of fresh RPMI 1640 supplemented with 0.1% BSA. The number of cells migrated across the filter to the lower chamber was determined by adding 20µl of Cell Titer 96 Aqueous One solution to each well of the plate as well as the standard curve. Cell Titer reagent works by viable cells bioreducing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium compound in the Cell Titer 96 Aqueous One solution leading to a colour change. Once the colour change had sufficiently developed the plates were then read at 490nm on a VersaMax platereader. Data was represented as a chemotactic index which is the number of cells migrated towards the ligand divided by the number of cells migrated towards vehicle. Basal migration is denoted by an index of 1.

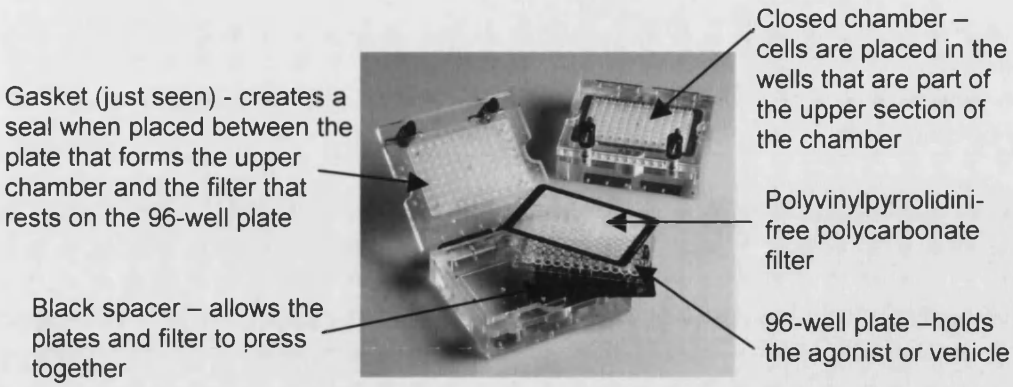


Figure 2.4: **Neuroprobe chemotaxis chamber.** Photo of the chamber obtained from the Neuroprobe website (www.neuroprobe.com).

2.8 Flow cytometry

2.8.1 Staining of cells with antibodies

Cells were washed twice in PBS and resuspended in ice cold PBS containing 20% (v/v) FBS (PBS/FBS). Primary antibody, diluted in PBS/FBS, was added to a total of $1x10^6$ cells/sample and the cells then rotated at 4°C. The final concentration of primary antibody depended on the individual antibody but was typically between 1-10μg/ml. After a 30 minute incubation period the cells were washed twice in ice cold PBS. The following stages were carried out in the dark. Secondary antibody was diluted in PBS/FBS to give a final concentration of 1:250 and added to the cells. Again the samples were rotated for 30 minutes at 4°C. They were then again washed twice in PBS and transferred to FACS tubes ready to be read. If pre-conjugated antibodies were used typically 20μl of antibody were added to $1x10^6$ cells in the dark as per manufacturers instructions and the cells allowed to rotate in the dark at 4°C. After a 30 minute incubation the cells were washed twice in ice cold PBS and put into FACS tubes. Whenever possible an isotype control was also set up to match the primary antibody. $1x10^6$ cells were also left untreated to give a measure of the cells' autofluorescence. All samples were read on a FACS Vantage (BD Biosciences, San Jose, CA) and analysed using the CellQuest programme.

2.8.2 Staining with phalloidin

Activated PBLs were set up as for stimulations, except always at a density of $2x10^6$ cells. In order to terminate the stimulations, rather than use lysis buffer to solubilise the cells, 3.7% formaldehyde was added to fix the cells. They were incubated in formaldehyde for 5 minutes at room temperature. The cells were then washed 2 times in PBS before being incubated in 0.1% (w/v) triton containing 0.1% (w/v) BSA to permeabilise the cells for 5 minutes at room temperature. The cells were then again washed twice in PBS to remove any traces of triton. The following steps were carried out in the dark. The cells were then incubated for 30 minutes with 0.6µM FITC-conjugated phalloidin and then washed 2 times in PBS. After the final wash, they were resuspended in 200µl of PBS and transferred to FACS tubes.

One set of unstimulated cells were always fixed and permeabilised as described above but not stained with phalloidin. These were used to set up the machine and determine the autofluorescence of the cells. All samples were read on a FACS Vantage (BD Biosciences, San Jose, CA) and analysed using the CellQuest programme. The median fluorescence intensity value for each sample was used in further data analysis.

2.9 Cell proliferation assays

2.9.1 XTT assay

Cell proliferation can be measured in a number of ways, including using XTT assays. The assay is set up such that cells are allowed to grow in a 96-well plate and at the end of the assay XTT, which is a yellow tetrazolium salt, is added to the cells. The assay works on the principle that the tetrazolium salt will be converted to an orange formazan dye by part of the respiratory chain of mitochondria and thus only in metabolically active cells. In this way a colour change can be measured and the optical densitometry will reflect the number of cells present in the wells. Thus the assay can be used to compare how different conditions affect cell number.

Activated PBLs were washed free of exogenous IL-2 the day prior to setting up the experiment but maintained in complete RPMI 1640. On the day the experiment was set up, cells were plated in 96-well tissue culture plates at a density of $5x10^4$ cells/well in complete RPMI 1640 and the appropriate concentration of cannabinoid and/or IL-2 added to the wells. After the incubation period, typically 48 or 72 hours, at 37°C in 5% CO_2, 25µl of PMS-XTT solution (1mg/ml XTT in RPMI 1640, containing 5µl/ml XTT in a solution of phenazine methosulfate which was made up in PBS at 1.53mg/ml) was added to each well and the plate returned to the incubator for a period of 2-6 hours to allow the plate to develop. The plate was then read on a VersaMax platereader at 450nm.

2.9.2 Counting cells using a Coulter counter

Activated PBLs were washed free of exogenous IL-2 the day prior to setting up the experiment but maintained in complete RPMI 1640. On the day the experiment was set up, cells were counted and placed in a 24 well plate at a density of $1x10^5$ cells/well in complete RPMI 1640 and the appropriate concentration of cannabinoid and/or IL-2 added to each well. After the incubation period, typically 72 hours, at 37°C in 5% CO_2, each sample was removed from the well and placed in isoton. The well was then washed out with an additional 500µl of media and this too was added to the isoton. The sample was then read twice on a Coulter Multisizer II (Beckman Coulter) in order to determine the number of cells in the sample.

2.10 Treatment of HT-29s with tunicamycin

Tunicamycin is a nucleoside antibiotic that prevents *N*-glycosylation from occurring in the endoplasmic reticulum and in this study was used to investigate whether the CB_1R is *N*-glycosylated.

HT-29s were set up in 6-well plates at a density of $0.3x10^6$ cells/well in 1.9ml complete McCoy's 5A and allowed to adhere overnight. The following day 100µl of tunicamycin or DMSO as vehicle control was added to each well to

obtain the appropriate final concentration. The cells were maintained at 37°C in an atmosphere of 5% CO_2 for the up to 48 hours, after which they were lysed in 100µl of lysis buffer/well. Samples were assayed for protein content and immunoblotted with the CB_1R antibody as described above.

2.11 Determination of intracellular cAMP

Intracellular cAMP content of activated PBLs was determined using a cAMP immunoassay (R&D Research). The assay functions by the cAMP present in the sample competing with a fixed amount of alkaline phosphatase-labeled cAMP for sites on a rabbit polyclonal antibody. This rabbit antibody is allowed to bind a goat anti-rabbit antibody which is coated on the plates provided in the kit. After removing excess conjugate and unbound sample, a substrate solution is added to each well to determine the bound enzyme activity. A change in colour occurs and the intensity of the colour is inversely proportional to the concentration of cAMP in the sample.

The protocol was followed as per manufacturers instructions. Briefly, activated PBLs were set up as for other stimulations at a total of $2x10^6$ per sample and allowed to rest in a 37°C water bath for 1 hour. The cells were then stimulated with cannabinoids and/or forskolin for the appropriate length of time. The reaction was terminated by rapidly spinning down the cells at 10,000rpm, removing the media and adding 255µl of 0.1N HCl/0.2% triton as provided in the kit. After a 20 minute incubation the samples were centrifuged and supernatants transferred to clean tubes. The reagents and standards were prepared and the plate set up as per the manufacturers instructions. The plates were read on a VersaMax platereader at 405nm.

2.12 Statistical analysis

Statistical analysis was performed where appropriate using One way analysis of variance (ANOVA) with Bonferroni correction where necessary or Student's *t* test. These were carried out using the GraphPad Prism version 3.0 software.

Chapter 3: Results I – Cannabinoid receptor protein expression in T lymphocytes

3.1 Background

It has been established that mRNA for both the CB_1R and CB_2R is expressed in a variety of immune cells (Bouaboula *et al.*, 1993, Galiegue *et al.*, 1995). Initially it was believed that the CB_1R was expressed only in neuronal tissue but the development of more sensitive RT-PCR techniques revealed that CB_1R mRNA was expressed, albeit at much lower levels than in the brain, in several immune subpopulations, including T lymphocytes (Bouaboula *et al.*, 1993, Galiegue *et al.*, 1995). The CB_2R is classically considered to be the 'immune' cannabinoid receptor and CB_2R mRNA has been previously detected in human T lymphocytes, although at lower levels than in other immune subpopulations: B lymphocytes > NK cells ≥ monocytes > polymorphonuclear neutrophils > T lymphocytes (Galiegue *et al.*, 1995). However, data published concerning CB_2R protein expression in T lymphocytes is contradictory. Staining of human tonsil sections revealed CB_2R expression in B lymphocyte, but not T lymphocyte, enriched areas of the mantle of the secondary lymphoid follicles (Galiegue *et al.*, 1995) and binding assays showed that specific binding was absent from T lymphocyte-enriched areas such as the thymus (Lynn & Herkenham, 1994). However, the CB_2R has been detected on human T lymphocytes using flow cytometry (Carayon *et al.*, 1998) and on T lymphocytes in mouse atherosclerotic lesions by immunoflourescent staining (Steffens *et al.*, 2005b). This part of the current study aimed to gain more definitive insight into the expression of the cannabinoid receptor proteins in T lymphocytes and hopefully dispel confusion from previous studies. Thus, using immunoblotting, a survey of CB_1R and CB_2R expression in normal human T lymphocytes and leukaemic T lymphocyte cell lines was carried out.

3.2 CB_1R and CB_2R protein expression in T cell lines

The CB_1R is only weakly expressed in immune cells (Galiegue *et al.*, 1995), thus it was necessary to establish which cell type could be used as a positive control for CB_1R protein expression before investigating its presence in T lymphocytes. Work carried out within the group (Wright *et al.*, 2005) showed that the colonic epithelial cell line, HT-29s, expressed the CB_1R, and therefore

they were, given that they were readily available to use, used as a control for CB_1R expression.

The predicted weight of the CB_1R is 53kD based on its amino acid sequence (Gerard *et al.*, 1991) and using the Cayman Chemicals CB_1R antibody a band of approximately this molecular weight was detected in WCLs of HT-29s (Fig. 3.1*A*) corroborating previous data (Wright *et al.*, 2005). In addition to the strong band at 53kD, a lighter band could sometimes also be detected just below, as in Fig. 3.1*A*. The antibody also recognised an 83kD band, which is generally presumed to be a modified form of the receptor resulting from post-translational modifications (Song & Howlett, 1995, Daaka *et al.*, 1996, Pettit *et al.*, 1998, Matias *et al.*, 2002) and which will be discussed in further detail below. In order to verify the specificity of the Cayman Chemicals CB_1R antibody in detecting these bands the appropriate blocking peptide was used. As the 53kD band was completely abolished and the 83kD band significantly reduced with the use of the blocking peptide (Fig. 3.1*C*) it suggests that both are forms of the CB_1R. In the HT-29 sample in Fig. 3.1*A* an additional faint band at approximately 60kD was also detected. This signal was not consistently detected in HT-29s but may also represent another post-translationally modified protein (Song & Howlett, 1995). No other immunoreactive bands were ever detected. Using the Cayman Chemicals CB_1R antibody only the 83kD band was detected in Jurkats and another leukaemic T lymphocyte cell line, CEMs (Fig. 3.1*A*).

The CB_2R is a 39kD protein and using the Cayman Chemicals CB_2R antibody an immunoreactive band of this approximate size was detected in Jurkat and also CEM WCLs (Fig. 3.1*B*). Previous studies have shown that Jurkats express CB_2R mRNA (Schatz *et al.*, 1997, McKallip *et al.*, 2002a) and thus it was not surprising to find that the Jurkats used in this investigation express CB_2R protein. Again, the use of a blocking peptide confirmed the specificity of the antibody in detecting this band (Fig. 3.1*D*). Having established that Jurkats express the CB_2R protein, WCLs were used as positive controls for CB_2R protein expression in the majority of subsequent experiments.

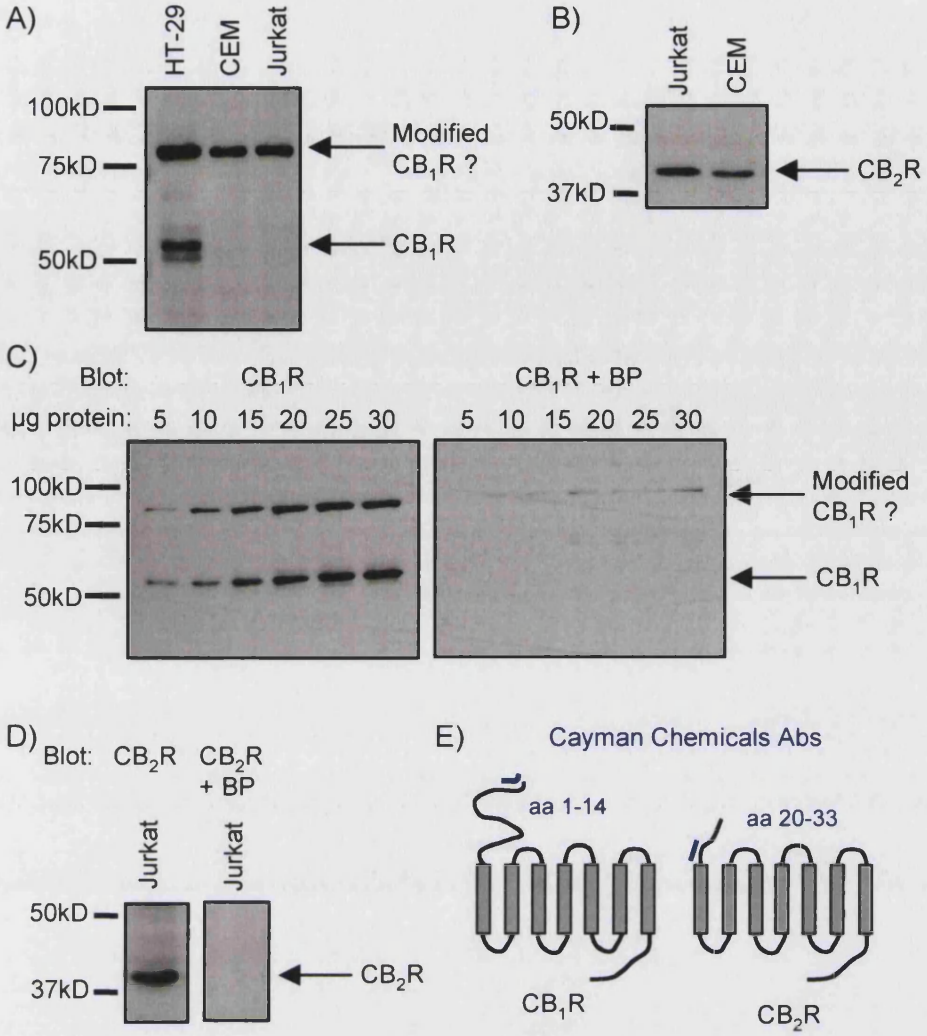


Figure 3.1: **CB_1R and CB_2R protein expression in HT-29s, Jurkats and CEMs.** Immunoblotting was used to determine the presence of CB_1R and CB_2R protein in the colonic epithelial cell line, HT-29s, and two leukaemic T lymphocyte cell lines, Jurkats and CEMs. *A* and *B*) WCLs were generated and proteins quantified by Bradford assay, boiled with 5x sample buffer, resolved by SDS-PAGE (30µg of protein was loaded), transferred to nitrocellulose membranes and immunoblotted, as described in *Materials and Methods*, with a polyclonal antibody raised against the N-terminus of the human CB_1R (*A*; Cayman Chemicals) or CB_2R (*B*; Cayman Chemicals). *C* and *D*) In order to verify the specificity of the antibodies, duplicate WCL samples of HT-29s (*C*) and Jurkats (*D*) were resolved by SDS-PAGE (30µg of protein was loaded) and the proteins transferred to nitrocellulose membranes. After the blocking stage these were cut in half and one half treated with the CB_1R (*C*) and the CB_2R (*D*) antibody as normal and the other half treated with antibody that had been incubated with the appropriate blocking peptide (BP; 1:9 for CB_1R and 1:4 for CB_2R). They were then further immunoblotted as described in *Materials and Methods*. Data are representative of at least three different sets of cells. *E*) Schematic representation of the CB_1R and CB_2R indicating which sections of the receptor the Cayman Chemicals antibodies were raised against.

3.3 Evidence suggesting the 83kD protein detected is a modified form of the CB_1R protein

Although the 83kD band detected by the Cayman Chemicals CB_1R antibody in HT-29s appears not to be due to non-specific interactions as preadsorbing of the antibody with its blocking peptide eliminated most of the signal, it is not clear exactly what this band represents. In the literature it is generally assumed to be a post-translationally modified form of the CB_1R (Pettit *et al.*, 1998, Wright *et al.*, 2005). However, as the 83kD band was also detected in Jurkats (Fig. 1*A*), which have previously been shown to not express CB_1R mRNA (Bouaboula *et al.*, 1993, Daaka *et al.*, 1996, Schatz *et al.*, 1997, McKallip *et al.*, 2002a) this 83kD protein was further investigated.

First, a negative control was carried out whereby membrane was probed only with the secondary antibody (data not shown). No bands were detected indicating that the 83kD band is not simply due to non-specific binding of the secondary antibody. A BLAST network search was also performed using the ExPASy Proteomics Server of the Swiss Institute of Bioinformatics to investigate whether the Cayman Chemicals CB_1R antibody could be cross-reacting with any other known proteins. The results indicate that it would only potentially cross-react with other CB_1R orthologues and the CB1B splice variant which is a truncated form of the receptor (Ryberg *et al.*, 2005), therefore suggesting that the 83kD protein detected is a form of the CB_1R.

Next, it was determined whether the CB_1R could be detected in Jurkats using other CB_1R-specific antibodies. If it was, this would further imply that the 83kD protein detected is indeed a form of the CB_1R protein. Both the Affinity Bioreagents and Santa Cruz antibodies used are, like the Cayman Chemicals antibody, raised against the N-terminus of the human CB_1R (see *Appendices* for more details on all three antibodies). The Affinity Bioreagents CB_1R antibody detected both a 53kD and higher, approximately 75kD, band in HT-29s (Fig. 3.2*A*), although the signal with this antibody was much weaker than that with the Cayman Chemicals antibody. Only the 75kD band was detected in Jurkats and

CEMs (Fig. 3.2*A*). The Santa Cruz CB_1R antibody detected a band at approximately 60kD in both HT-29s and Jurkats (Fig. 3.2*B*). These results further indicate that the Jurkats used in this study do express CB_1R protein and hence that the 83kD protein detected by the Cayman Chemicals antibody represents a form of the receptor rather than an artefact.

One of the questions raised is whether the 83kD protein is expressed at the cell surface. Although immunoblotting of WCLs is an indication that a protein is expressed in the cell it does not determine its intracellular localisation. Cytosolic and membrane fractions of Jurkats and HT-29s were generated and protein expression examined using the Cayman Chemicals receptor antibodies in order to investigate where the 83kD protein is expressed. Both fractions of the HT-29s express the 53kD and 83kD proteins (Fig. 3.3*A*). Unfortunately, as the samples were loaded according to equal quantities of protein rather than equal cell number it cannot be concluded which fraction contained more or less of each protein. Equally, the CB_2R is also expressed in both fractions of Jurkats, as is the 83kD form of the CB_1R (Fig 3.3*B*). In each instance samples were also blotted for IκBα, a cytosolic protein involved in sequestering NFκB proteins in the cytoplasm, preventing their activation (Viatour *et al.*, 2005, Campbell & Perkins, 2006), in order to verify the purity of the fractions (Fig. 3.3). In each sample the IκBα protein was only detected in the cytosolic fraction and weakly in the WCL but not in the membrane fractions indicating that the fractions were pure. Thus the 83kD protein can be membrane localised indicating that it is likely to be expressed at the cell surface.

Given its molecular weight, the 83kD protein is presumably a post-translationally modified form of the CB_1R. Indeed, this is what is generally stated in papers (Song & Howlett, 1995, Daaka *et al.*, 1996, Pettit *et al.*, 1998, Matias *et al.*, 2002). The rat brain CB_1R protein was found to be *N*-glycosylated at two out of three potential sites at the N-terminus to produce 59kD and 64kD proteins (Song & Howlett, 1995). Thus, the question raised is whether the 83kD protein represents a more heavily *N*-glycosylated form of the CB_1R. Tunicamycin, which is a nucleoside antibiotic that prevents *N*-glycosylation from occurring in the endoplasmic reticulum, was used to investigate this. HT-29s were treated

with tunicamycin or an equivalent concentration of vehicle for up to 48 hours. There was no noticeable change in expression of the 83kD band over the time course or with varying concentrations of tunicamycin (Fig. 3.4*A* and *B*). However, the 53kD band was decreased in both a time and concentration-dependent manner (Fig. 3.4*A* and *B*). The intensity of the 53kD band began to decrease after 8 hours of treatment and although the effect was strongest at 10μg/ml, 1μg/ml also decreased levels of the 53kD protein.

This decrease in the level of the 53kD protein was unexpected given that 53kD is the calculated molecular weight of the protein based solely on its amino acid sequence and it raised the question of whether the 53kD band that is detected by the Cayman Chemicals antibody is actually a 53kD protein. In some blots, such as Fig. 3.1*A*, it is possible to detect an additional band just below the supposed 53kD band and it is likely that this lower band is usually masked by the stronger signal of the band above. It is possible that it is this lower band is actually the 53kD protein and that the higher band referred to up until now as being 53kD is actually 55-56kD and represents a *N*-glycosylated CB_1R. Unfortunately in the blots relating to the tunicamycin experiments it was not possible to clearly detect both bands and confirm this. To test whether the two bands would be easier to distinguish on a 12% SDS-PAGE gel compared to the 10% gels normally used, WCLs of untreated HT-29s were resolved on 12% gels. Fig. 3.4*C* shows that on 12% gels the two bands are easier to distinguish and thus these could be used to investigate whether tunicamycin only affects the expression of the higher band or not. As the nature of these two bands has not been concretely resolved the strong signal that is detected at approximately 53kD will continue to be referred to as the 53kD band throughout the rest of this study.

Collectively, these results suggest that the 83kD protein detected by the Cayman Chemicals antibody is a modified form of the CB_1R and that it can be expressed at the cell surface alongside the 53kD CB_1R protein. Results indicate that the 83kD protein in not *N*-glycosylated and further investigation is needed to elucidate exactly how it is modified. Furthermore, it was established that the Jurkats used in this study also express a form of the CB_1R.

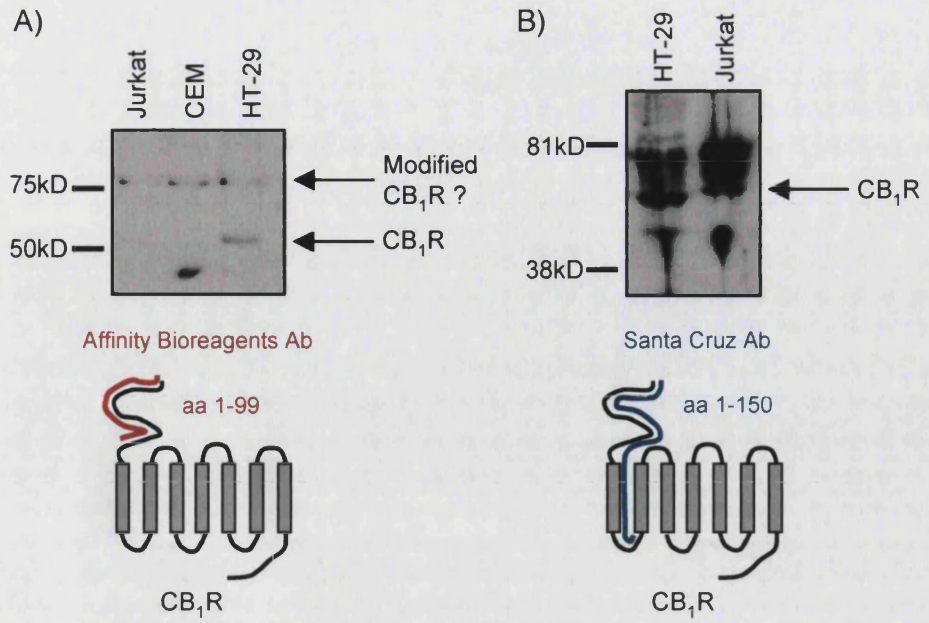


Figure 3.2: **Detection of the CB_1R protein using alternative antibodies.** Two additional, commercially available, CB_1R antibodies were used to investigate whether the 83kD protein detected using the Cayman Chemicals antibody is a modified form of the receptor. WCLs of the various cell types were generated as described in *Materials and Methods*. The proteins were quantified by Bradford assay, samples boiled with 5x sample buffer, resolved by SDS-PAGE (30µg of protein was loaded), transferred to nitrocellulose membranes and immunoblotted with antibodies raised against the human CB_1R. *A*) Affinity Bioreagents, representative of a single experiment, *B*) Santa Cruz, representative of two different sets of cells. Schematic representations of the CB_1R indicating which sections of the receptor each antibody is raised against have been included.

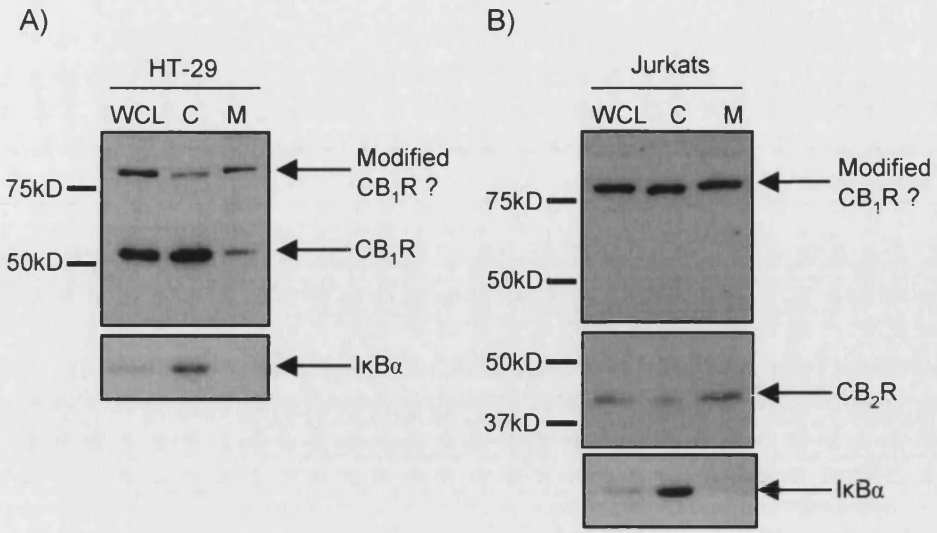


Figure 3.3: **Localisation of the CB_1R and CB_2R within cells.** HT-29s (*A*) and Jurkats (*B*) were lysed as described in *Materials and Methods* to generate cytosolic (C) and membrane (M) fractions and WCLs. Samples were assayed for protein content by Bradford assay, boiled with 5x sample buffer, resolved by SDS-PAGE (30µg of protein was loaded), transferred to nitrocellolose membrane and immunoblotted, as described in *Materials and Methods*, with antibodies against the CB_1R or CB_2R (Cayman Chemicals). Samples were also immunoblotted with an antibody to IκBα in order to verify the purity of the fractions. Data are representative of two different sets of cells.

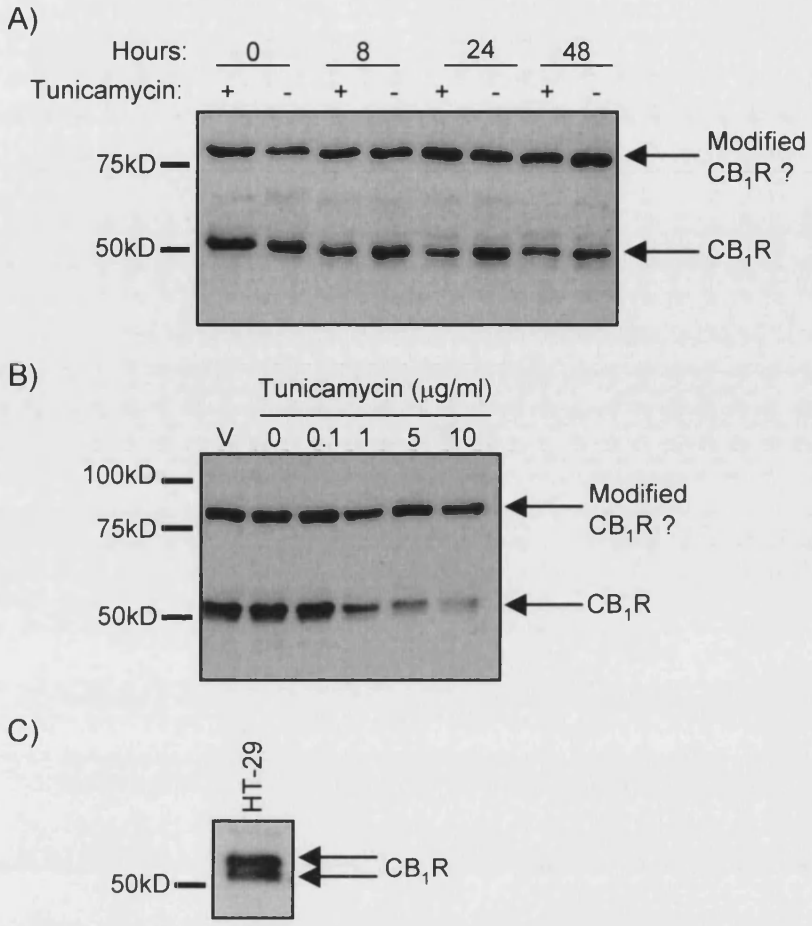


Figure 3.4: **The effect of tunicamycin on CB_1R protein expression in HT-29s.** *A* and *B*) HT-29s were set up in 6-well plates as described in *Materials and Methods* and treated either with 1μg/ml tunicamycin (+) or vehicle (-; DMSO) for increasing lengths of time (*A*) or with (0-10μg/ml) or vehicle (V; DMSO equivalent to 10μg/ml tunicamycin) for 48 hours (*B*). At the end of the incubation period WCLs were generated and proteins quantified by Bradford assay, boiled with 5x sample buffer, resolved by SDS-PAGE (30μg of protein was loaded), transferred to nitrocellulose membranes and immunoblotted, as described in *Materials and Methods*, with an antibody raised against the human CB_1R (Cayman Chemicals). Data are representative of three different sets of cells. *C*) WCLs of HT-29s were generated and resolved on 12% SDS-PAGE gels (30μg of protein was loaded), transferred to nitrocellulose membranes and immunoblotted, as described in *Materials and Methods*, with an antibody raised against the human CB_1R (Cayman Chemicals). Data are representative of two different sets of cells.

3.4 CB_1R and CB_2R protein expression in naïve T lymphocytes and PBLs

T lymphocytes express both CB_1R and CB_2R mRNA (Galiegue *et al.*, 1995) and although one study has indicated murine T lymphocytes express CB_1R protein (Klein *et al.*, 2003) data regarding CB_2R protein expression is contradictory (Lynn & Herkenham, 1994, Galiegue *et al.*, 1995, Carayon *et al.*, 1998, Steffens *et al.*, 2005b). One of the main aims of this project was to establish which cannabinoid receptors are expressed in human T lymphocytes, starting with naïve peripheral T lymphocytes. Cells were isolated from whole human blood as described in *Materials and Methods* and WCLs generated. Using the Cayman Chemicals CB_1R antibody only the 83kD form of the CB_1R was detected in naïve human T lymphocytes (Fig. 3.5*A*). The CB_2R protein was not detected in these cells (Fig. 3.5*B*).

Previous studies have reported that cannabinoid receptor expression can be upregulated or downregulated by a variety of factors such as differentiation as in the case of B lymphocytes (Carayon *et al.*, 1998, Marchand *et al.*, 1999). However, these investigations have focused on the changes in receptor mRNA expression over 24 or 48 hours and have not examined the long-term effects on protein levels. In order to explore the long-term changes elicited by activation on cannabinoid receptor expression in T lymphocytes, PBLs were generated. PBLs are T lymphocytes that have been activated *in vitro*, for instance by the superantigen SEB, and clonally expanded using IL-2. Cells were removed from culture at day 5 and 12 after initial PBMC isolation and stained for CD3, CD4 and CD8 as described in *Materials and Methods* in order to verify that a mainly T lymphocyte population was generated and that there was no or little contamination from B lymphocytes or monocytes. The results confirm that the method of PBL generation used yielded an almost pure T lymphocyte population, as measured by CD3 expression, which was approximately 80% $CD4^+$ (Table 3).

	$CD3^+$	$CD4^+$	$CD8^+$
Day 5	97.38 (±2.99)	80.93 (±4.85)	24.29 (±7.06)
Day 12	97.88 (±0.70)	76.02 (±12.68)	28.06 (±11.83)

Table 3: **Characterisation of activated PBLs.** Cells were removed from culture at days 5 and 12 after initial PBMC isolation and stained using fluorescently labelled CD3, CD4 and CD8 antibodies as described in *Materials and Methods*. Fluorescence was detected using flow cytometry and data are represented as the percentage of the population expressing each marker. Data are stated as mean ±S.E.M, n=3.

The CB_2R was not detected in the freshly isolated PBMCs used to generate the PBLs, however, expression of the CB_2R protein was upregulated following T lymphocyte activation with SEB (Fig. 3.6*B*). This upregulation was transient, with receptor expression typically being downregulated around day 7-8. Expression consistently peaked at around day 5 of culture. WCLs of day 5 PBLs, when CB_2R expression was high, and day 12 PBLs, when expression was downmodulated, were also probed with an additional CB_2R antibody to verify that these differences in expression could be detected by more than one antibody. The Santa Cruz CB_2R antibody, which is raised against the C-terminus of the CB_2R (further details on both antibodies can be found in *Appendices*), detected an approximately 60kD band in Jurkats and CEMs as well as in day 5 PBLs (Fig. 3.6*B*). Expression was much lower in day 12 PBLs as seen with the Cayman Chemicals antibody. Only the 83kD form of the CB_1R protein was detected in both PBMCs and PBLs and its expression remained constant throughout the culture period (Fig. 3.6*A*).

In addition to activating T lymphocytes using the superantigen SEB, PBMCs were also activated using CD3/CD28-coated beads in order to study the effect of different types of stimulus on cannabinoid receptor protein expression. Again, only the modified 83kD CB_1R protein was detected and its expression was not altered during the course of the culture (Fig. 3.6*E*). However, the CB_2R was again upregulated following T lymphocyte activation and the elevated levels of expression induced were maintained for the duration of the culture period (Fig. 3.6*F*). Interestingly, in the cultures used for these experiments, some CB_2R was detected in the PBMC population contrary to those used in the SEB-activated PBL experiments.

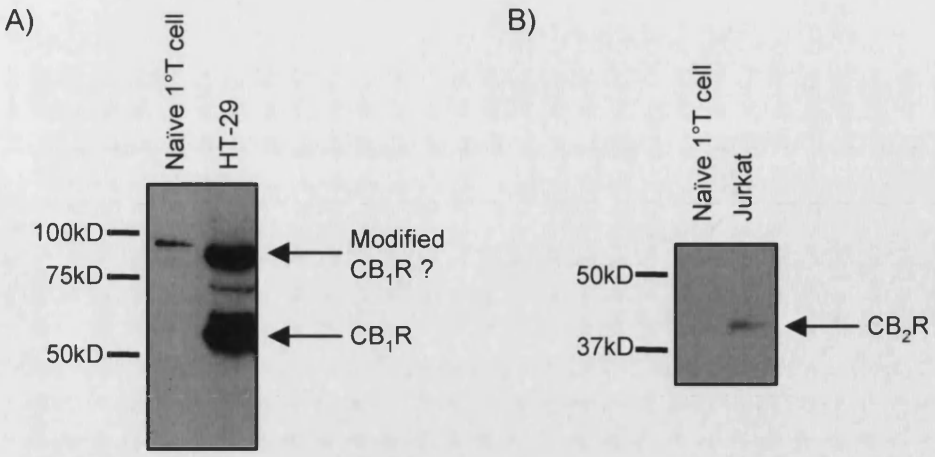


Figure 3.5: **CB_1R and CB_2R protein expression in primary naïve T lymphocytes.** Immunoblotting was used to determine the presence of the CB_1R and CB_2R in naïve T lymphocytes. These were purified from human PBMCs as described in *Materials and Methods* and WCLs generated. Proteins were quantified by Bradford assay, boiled with 5x sample buffer, resolved by SDS-PAGE (30µg of protein was loaded), transferred to nitrocellulose membranes and immunoblotted, as described in *Materials and Methods*, with antibody raised against the human CB_1R (*A*; Cayman Chemicals) or CB_2R (*B*; Cayman Chemicals). HT-29 and Jurkat WCLs were used as positive controls for CB_1R and CB_2R protein expression, respectively. Data is representative of three different sets of cells.

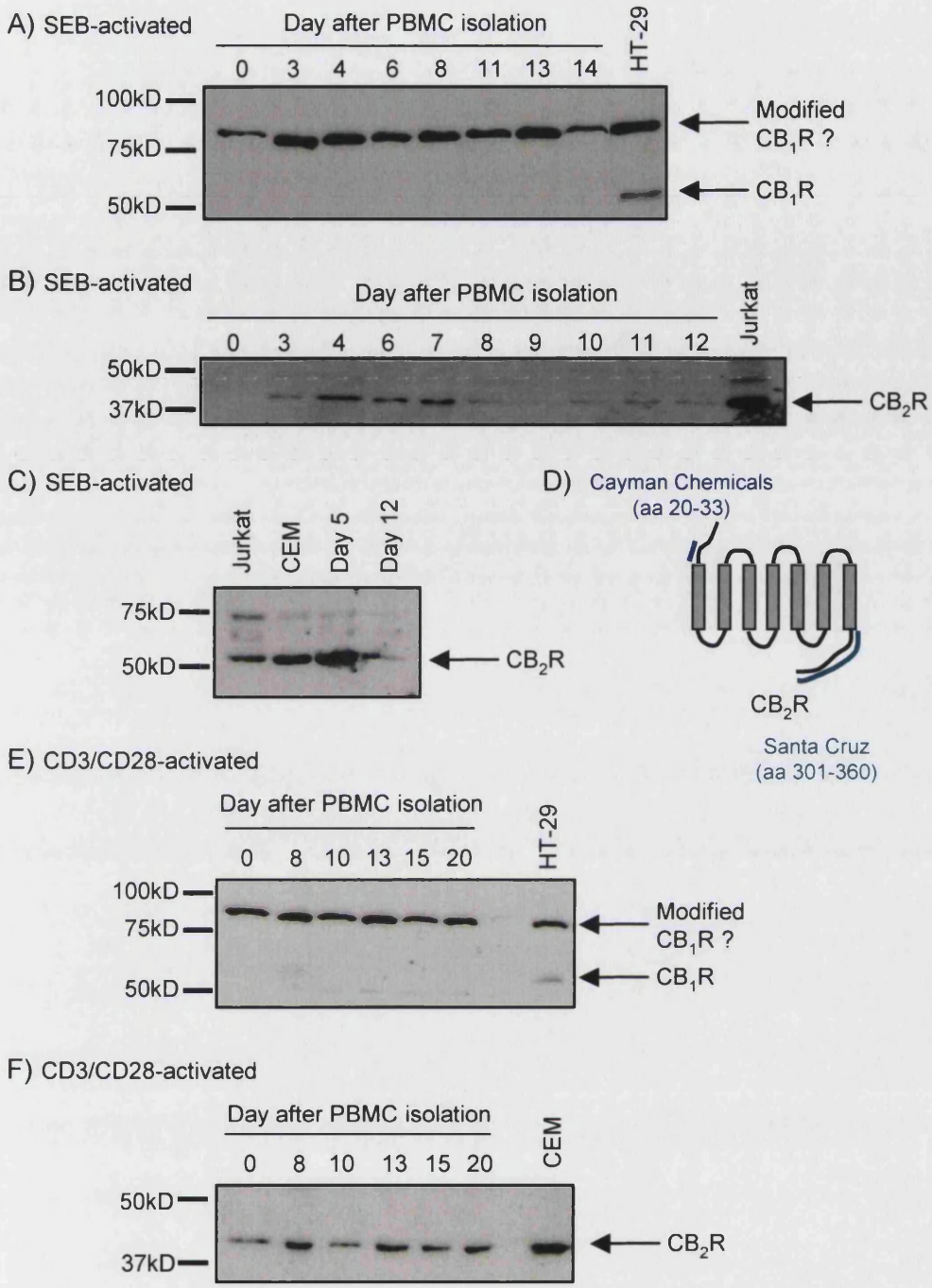


Figure 3.6: **CB_1R and CB_2R protein expression in activated PBLs.** Immunoblotting was used to determine the presence of CB_1R and CB_2R protein in PBLs throughout the culture period. PBMCs were isolated from human blood and the T lymphocytes activated using SEB (*A-C*) or CD3/CD28-coated beads (*E, F*) as described in *Materials and Methods*. Samples of cells were removed from culture on different days post-isolation and WCLs generated. Protein levels were quantified by Bradford assay, samples boiled with 5x sample buffer, resolved by SDS-PAGE (30µg of protein was loaded), transferred to nitrocellulose membranes and immunoblotted, as described in *Materials and Methods*, with antibody raised against the human CB_1R (*A, E*; Cayman Chemicals) and CB_2R (*B, F*, Cayman Chemicals; *C*, Santa Cruz). HT-29 and Jurkat WCLs were used as positive controls for CB_1R and CB_2R protein respectively. Data are representative of at least three (*A* and *B*) or two (*C, E, F*) different sets of cells. *D*) Schematic representation of the CB_2R showing which sections of the receptor the antibodies are raised against.

3.5 Potential heterodimerisation between the CB_1R and CB_2R

Although the existence of CB_1R homodimers has been suggested in several papers (Wager-Miller *et al.*, 2002, Mackie, 2005, Xu *et al.*, 2005), thus far no one has reported whether the CB_1R and CB_2R dimerise. As the CB_1R has been shown to dimerise with several GPCRs including the LPA1 receptor (Wright *et al.*, 2005) and D2 dopamine receptor (Kearn *et al.*, 2005) it would not be surprising to find that the CB_1R and CB_2R could form heterooligomers in cells where they are both expressed. Given the impact that dimerisation is able to have on, for instance, receptor pharmacology (Maggio *et al.*, 2005), and that activated PBLs appear to express both the CB_2R and a form of the CB_1R, the possibility of CB_1R-CB_2R dimerisation was investigated in some preliminary experiments.

As HT-29s have been shown to express both the 53kD form of the CB_1R and also the CB_2R (Ihenetu *et al.*, 2003b, Wright *et al.*, 2005) dimerisation was investigated in this cell line. Preliminary data collected suggests that these two receptors can indeed associate (Fig. 3.7). Anti-CB_1R IPs were generated using the Santa Cruz CB_1R antibody and resolved by SDS-PAGE and membranes probed with the Cayman Chemicals CB_2R antibody. The CB_2R was detected in the HT-29 WCL sample as well as in the IP sample, suggesting an association between the two receptors. It remains to be seen whether the 83kD protein and the CB_2R could also dimerise.

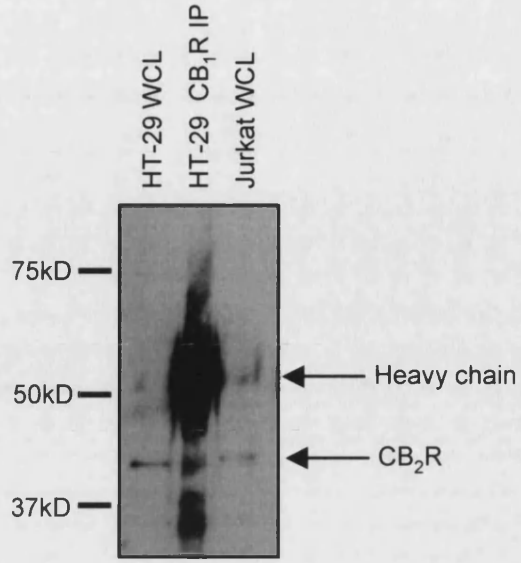


Figure 3.7: **Preliminary evidence suggesting CB_1R-CB_2R dimerisation in HT-29s.** HT-29s were grown on 10cm dishes, lysed and immunoprecipitated using CB_1R antibody (Santa Cruz) as described in *Materials and Methods*. WCLs of HT-29s and Jurkats were also generated at the same time. Samples were boiled with sample buffer, resolved on 12% SDS-PAGE gels (30µg of protein was loaded except for the IP sample which was loaded in its entirety), transferred to nitrocellulose membrane and immunoblotted as described in *Materials and Methods*, with antibody against the CB_2R (Cayman Chemicals). Data is representative of 2 separate experiments.

3.6 Expression of FAAH and MAGL in T lymphocytes

As well as the receptors and the endogenous ligands, the cannabinoid system also comprises the enzymes involved in metabolising the endocannabinoids. These include FAAH, which primarily breaks down AEA but can also act on 2-AG (Goparaju *et al.*, 1998, Cravatt & Lichtman, 2002), and MAGL, which metabolises 2-AG (Dinh *et al.*, 2002). It is possible that some of the effects contributed to the endocannabinoids may in part be due to their metabolites (Pertwee & Ross, 2002) and thus it is important to establish whether these two enzymes involved in endocannabinoid metabolism are expressed in the primary T lymphocytes and leukaemic T lymphocyte cell lines used in this study.

FAAH is a 63kD protein and has previously been detected in human T lymphocytes (Maccarrone *et al.*, 2003c). Immunoblotting with an antibody raised against human FAAH detected the expression of FAAH protein in day 5 activated PBLs as well as the Jurkats and CEMs used in this study (Fig. 3.8). Interestingly, FAAH appeared as a doublet and ran at a slightly higher weight in the Jurkats and CEMs than in the PBLs, suggesting that in these cell lines FAAH may undergo some post-translational modifications. MAGL, a 33kD protein (Dinh *et al.*, 2002), was also detected also in CEMs, Jurkats and day 5 activated PBLs (Fig. 3.8). Notably, these blots only confirm the expression of these proteins, they cannot be used to compare amounts of enzyme expressed in each cell type as different amounts of protein were loaded per well.

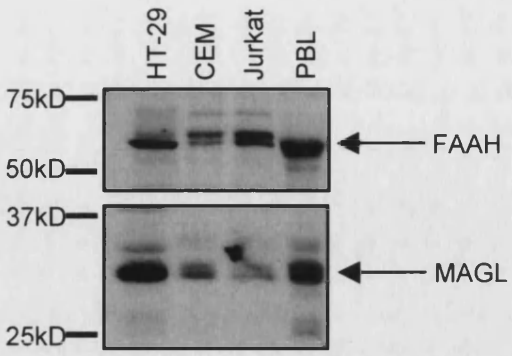


Figure 3.8: **FAAH and MAGL expression in HT-29s, CEMs, Jurkats and PBLs.** Immunoblotting was used to determine the presence of FAAH and MAGL in several different cell types including day 5 activated PBLs. WCLs were generated and proteins were quantified by Bradford assay, boiled with 5x sample buffer, resolved by SDS-PAGE (20-30µg of protein was loaded per well), transferred to nitrocellulose membranes and immunoblotted, as described in *Materials and Methods*, with antibodies raised against human FAAH and MAGL. Data are representative of two separate experiments.

3.7 Summary

- Using *in vitro* activated human T lymphocytes or PBLs this study has shown that the CB_2R protein is upregulated following T lymphocyte activation. The mode of activation appears to influence how long the CB_2R is upregulated for.
- In addition, a 83kD form of the CB_1R is also expressed in T lymphocytes although it is not influenced by cellular activation. The exact nature of this 83kD protein remains unknown.
- Preliminary data suggests that the two cannabinoid receptors may be able to dimerise in cells where they are both expressed.
- It was also found that T lymphocytes express both FAAH and MAGL, two of the enzymes involved in the breakdown of the endocannabinoids AEA and 2-AG.

3.8 Discussion

One of the main aims of this part of the study was to gain insight into the expression of the cannabinoid receptor proteins in T lymphocytes. This involved not only investigating CB_1R and CB_2R protein expression in naïve peripheral T lymphocytes but also in activated T lymphocytes. However, before this was work was carried out, expression in T lymphocyte cell lines was determined as a comparison.

3.8.1 CB_1R protein expression in leukaemic T lymphocyte cell lines

3.8.1.1 The 53kD CB_1R is not detected in Jurkats or CEMs

Using the Cayman Chemicals CB_1R antibody, which is raised against the N-terminus of the human CB_1R, a strong immunoreactive band of approximately 53kD was detected in HT-29s, a colonic epithelial cell line. This corresponds to the calculated molecular weight of the CB_1R based on its amino acid sequence and confirms previous data generated in the lab (Wright *et al.*, 2005). The

specificity of the antibody in detecting this band was confirmed through use of the appropriate blocking peptide. Having established that HT-29s express the 53kD CB_1R protein they were used as positive controls for the CB_1R protein in further experiments. This 53kD band was not detected in Jurkats, nor in CEMs, another leukaemic T lymphocyte cell line.

In addition to the 53kD band, an approximately 60kD band was also sometimes detected in HT-29s using the Cayman Chemicals CB_1R antibody. The rat CB_1R was shown to be *N*-glycosylated at two out of three potential sites in the N-terminus generating 59kD and 64kD proteins (Song & Howlett, 1995). The human CB_1R also has three potential *N*-glycosylation sites (Gerard *et al.*, 1991) and thus this band is presumably an equivalent *N*-glycosylated form. It is likely that the lack of reliability in detecting this band in HT-29s is related to low expression and hence whether it is detected depends on the sensitivity of the antibody in any given experiment. It was also not detected in either Jurkats or CEMs. Although the Cayman Chemicals antibody would be able to detect the CB1B which is a truncated splice variant of the CB_1R (Ryberg *et al.*, 2005) no appropriately sized bands were detected in HT-29s or any of the other cell types used in this investigation.

Several papers have also reported the detection of larger proteins using immunoblotting and suggest that these indicate that the CB_1R can form homodimers or larger oligomers (Pettit *et al.*, 1998, Wager-Miller *et al.*, 2002, Mackie, 2005, Xu *et al.*, 2005). For instance, Wager-Miller *et al* have detected CB_1R homodimers or larger oligomers using a C-terminal CB_1R antibody which they say preferentially recognises the dimerised form of the receptor (Wager-Miller *et al.*, 2002, Mackie, 2005). Using this antibody a 160-200kD band was detected in rat membranes (Wager-Miller *et al.*, 2002, Mackie, 2005) which was suggested to be either SDS-resistant CB_1R homodimers migrating anomalously or larger aggregates. The antibody labelled the same rat brain areas as the N-terminal CB_1R antibodies and failed to label any areas in CB_1R knockout animals, suggesting that it is indeed CB_1R specific (Hajos *et al.*, 2000, Katona *et al.*, 2001). The authors also say that they have shown CB_1R dimer formation

using immunoprecipitation but this data has not yet been published (Mackie, 2005).

Further suggestion that the CB_1R exists as homodimers has come from a study investigating human CB_1R protein purified from a baculovirus expression system (Xu *et al.*, 2005). As well as a monomeric CB_1R protein, a larger 120kD band was also detected by immunoblotting using an N-terminal CB_1R antibody and was found to consist of CB_1R monomers, thus confirming the existence of SDS-resistant CB_1R homodimers in this expression system. Other papers using N-terminal CB_1R antibodies have also detected proteins of approximately 116-120kD, for example in rat brain (Pettit *et al.*, 1998), DLD-1 human colonic epithelial cells (Ligresti *et al.*, 2003) and mouse small intestine (Casu *et al.*, 2003) and 160-200kD, for instance in rat brain membrane (Mukhopadhyay *et al.*, 2000). Again, these would suggest the existence of SDS-resistant CB_1R homodimers or larger oligomers in tissues, rather than just in an expression system.

However, in the current study, using three different N-terminal CB_1R antibodies, bands of either 116-120kD or 160-200kD were not detected in HT-29s. This indicates that any CB_1R dimers in these cells are SDS-sensitive, in contrast to the SDS-resistant dimers suggested to exist by others. Although it is certainly likely that the CB_1R does form homodimers given that it is suspected that all GPCRs can dimerise (Hansen & Sheikh, 2004, Maggio *et al.*, 2005), further investigation is needed. Techniques such as size exclusion chromatography would allow a more accurate molecular weight of the 120kD/160-200kD complexes to be determined whilst, for example, purifying and sequencing the bands would resolve what the components are. Creating truncated receptors would also establish which parts of the receptor are involved in dimer formation and confirm whether the C-terminus is indeed involved as suggested by the CB_1R C-terminal antibody (Wager-Miller *et al.*, 2002).

3.8.1.2 An 83kD protein is detected in Jurkats and CEMs

Although CB_1R homodimers were not detected using the Cayman Chemicals CB_1R antibody, as well as the 53kD band, a strong band of approximately 83kD was detected. In the literature it is generally stated to be a post-translationally modified form of the receptor (Song & Howlett, 1995, Daaka *et al.*, 1996, Pettit *et al.*, 1998, Matias *et al.*, 2002) and there are several lines of evidence to support this notion which will be discussed below.

3.8.1.2.1 Evidence that the 83kD protein is a modified CB_1R

Firstly, use of the blocking peptide to preadsorb the antibody eliminated most of the signal, indicating that it is not simply non-specific binding and suggesting it is an additional form of the receptor. In addition, the 83kD band was not detected when a negative control was carried out by probing the membrane with only the secondary antibody, again confirming it is not due to non-specific binding. Recently, Klein *et al* (2003) reported that using commercial polyclonal antisera generated against CB_1R peptides they detected major bands of either 65kD or 75kD in splenocytes derived from wild type and CB_1R knockout mice, thus calling into question the specificity of the available CB_1R antibodies. Notably, they failed to specify which antibodies they used and also did not test one they generated against the 14 amino acids of the N-terminus of the CB_1R, like the Cayman Chemicals antibody, on samples from knockout mice. They suggest that one explanation is that the antibodies are detecting other proteins that share epitopes with the CB_1R. Therefore, a BLAST network search was carried out to verify whether the Cayman Chemicals antibody can react with any other know proteins. The search revealed that the antibody would only react with other CB_1R orthologues and the CB1B. Thus, it is likely that the 83kD band represents an additional form of the CB_1R protein rather than non-specific binding or another protein.

Secondly, only this 83kD protein was detected in Jurkat and other CB_1R antibodies were used to verify the presence of the receptor in these cells. Although the CB_1R has been suggested to be expressed on CEMs (Powles *et al.*,

2005), several reports have stated that Jurkats express either no (Bouaboula *et al.*, 1993, Schatz *et al.*, 1997, McKallip *et al.*, 2002a) or very little (Daaka *et al.*, 1996) CB_1R mRNA and no protein (Daaka *et al.*, 1996). Thus, initially it raised the possibility that either the 83kD band is in fact not a CB_1R or the Jurkats used in this study do express a CB_1R. Therefore, additional commercially available CB_1R antibodies were used in order to address this issue. Although the Affinity Bioreagents antibody appeared not to be as sensitive as the Cayman Chemicals antibody it also detected a 53kD band in HT-29s, but not Jurkats or CEMs, and a higher band in all three cell lines. The higher band ran at approximately 75kD on the gel rather than 83kD as seen with the Cayman Chemicals antibody but this may simply be an anomaly of the experiment. The Santa Cruz CB_1R antibody also detected a 60kD band although several additional smeared bands were also visible. The 60kD band was also detected in Jurkats, suggesting that the cells used in this study do express the CB_1R protein and hence that the 83kD band detected with the Cayman Chemicals antibody is a form of the receptor. One important point to make is that all three of the antibodies used were raised against the N-terminus of the CB_1R and therefore the band detected in Jurkats may simply be a result of all three antibodies detecting the same alternative protein. However, given that the BLAST search results revealed no such alternative protein it is very unlikely that this is the case. One way of confirming this would be to use the new commercially available CB_1R antibody which has been generated against amino acids 461-472 at the C-terminus of the receptor.

Daaka *et al* (1996) have shown that in Jurkats that express almost negligible amounts of CB_1R mRNA and no protein, receptor expression both at an mRNA and protein level can be upregulated by mitogen stimulation. In fact, the antibody used in this study detected several bands including ones at 45kD, 60kD and 87kD in N18TG2 mouse neuroblastoma cells which were used as a positive control and the 87kD band was amongst those upregulated upon mitogen stimulation. Thus it is possible that the Jurkats used in the current study have undergone some transformation to now consistently and strongly express the CB_1R. This data does underline the need to be careful when interpreting data from cell lines and to ensure that characteristics such as receptor expression are monitored.

Having determined that the 83kD protein detected by the Cayman Chemicals CB_1R antibody is a form of the receptor, one question raised is whether or not this protein is expressed at the cell surface. The CB_1R has previously been shown to be expressed at the plasma membrane and associates with lipid rafts (Sarnataro *et al.*, 2005). It has also been shown to be expressed in lysosomes (Sarnataro *et al.*, 2005) and can constitutively cycle between the plasma membrane and endosomes (Leterrier *et al.*, 2004). This, together with the fact that its long N-terminal tail cannot be efficiently translocated across the ER membrane (Andersson *et al.*, 2003), leads to low expression at the plasma membrane. Results obtained from generating cytosolic and membrane fractions of HT-29s and Jurkats indicate that the 83kD protein is membrane localised in both cell types, as well as being in the cytosol. Similarly, the 53kD CB_1R and the CB_2R were also detected in cytosolic and membrane fractions of HT-29s and Jurkats respectively, suggesting that they too are expressed at the cell surface.

Although the 83kD CB_1R protein is generally stated to be a post-translationally modified form of the receptor in the literature, to the best of my knowledge the nature of this modification has not been investigated. The human CB_1R has three potential *N*-glycosylation sites within in the N-terminus of the protein (Gerard *et al.*, 1991). An early study looking at rat CB_1R *N*-glycosylation only detected a 59kD and 64kD glycosylated form of the receptor suggesting that only two out of the three potential sites are actually *N*-glycosylated (Song & Howlett, 1995). Using the inhibitor tunicamycin it was investigated whether the 83kD protein detected using the Cayman Chemicals antibody is *N*-glycosylated. The 83kD protein was unaffected by tunicamycin treatment, even at high concentrations, indicating it is not *N*-glycosylated.

Surprisingly, the 53kD band in HT-29s was nearly abolished by tunicamycin treatment. This was unexpected as the 53kD band is expected to represent the unmodified CB_1R. However, in HT-29s the 53kD band is sometimes accompanied by an additional lower band as in Fig. 3.1*A* which is not always detected and may be masked by the intensity of the 53kD band as it runs very close to it. This slightly lower band may in fact represent the 53kD protein,

meaning that the slightly higher band is a *N*-glycosylated protein which is actually 55-56kD. The added weight may only be about 2kD suggesting a small oligosaccharide addition, which would not be surprising given that the three potential *N*-glycosylation sites are all very close together at residues 77, 83 and 112 (of the human CB_1R) and thus steric hindrance may prevent the addition of a large carbohydrate moiety at each of these sites. If this lower band is indeed the true, unglycosylated, 53kD protein it should not be affected by tunicamycin treatment. Unfortunately, it was not possible to clearly identify the lower band in any of the blots obtained from experiments were tunicamycin was used thus this could not be confirmed. However, results indicate that the two bands are easier to distinguish when HT-29 WCLs were resolved on 12% SDS-PAGE gels rather than the 10% gels routinely used. Thus, these higher percentage SDS-PAGE gels could be used to investigate whether or not tunicamycin only affects expression of the higher band. As for now it is not clear whether there are two bands or not the strong signal detected at approximately 53kD will continue to be referred to as the 53kD form of the CB_1R protein.

The data suggests that the 83kD protein is not *N*-glycosylated. There are many more types of post-translational modifications, however, given the size of the modification it is most likely that *O*-glycosylation would account for this large CB_1R protein. Although there is no specific consensus sequence for *O*-glycosylation as there is for *N*-glycosylation, there are many serine and threonine residues in the N-terminal domain (Gerard *et al.*, 1991) suggesting that the CB_1R could be *O*-glycosylated.

3.8.1.2.2 What else could the 83kD protein represent?

As the data collected did not determine what the post-translational modification is, one cannot exclude the possibility that the protein detected is not strictly a single, modified, CB_1R. When the human CB_1R was purified and expressed using a baculovirus expression system an 80kD protein was detected and it was shown that this protein consisted of two truncated CB_1Rs (Xu *et al.*, 2005). Therefore, the 83kD protein detected in this study may consist of two truncated CB_1R proteins. The receptors would have to be truncated at the C-terminus as

otherwise the Cayman Chemicals antibody, which is raised against the first 14 amino acids of the N-terminus, would not detect the complex. The complex would also have to be SDS-resistant. The protein is too small to be a CB_1R or CB1B homodimer and it is also too small to be a dimer of the CB_1R with one of the CB_1R splice variants, CB1A or CB1B (Shire *et al.*, 1995, Ryberg *et al.*, 2005). However, it may be complexed with other proteins. For instance, two cannabinoid receptor interacting proteins (CRIPs), CRIP1a and CRIP1b, have recently been identified (Niehaus *et al.*, 2004). These proteins bind to the C-terminal tail of the CB_1R and CRIP1a binding has been proposed to decrease the receptors' constitutive activity (Niehaus *et al.*, 2004). Although these proteins are likely too small to account for the 83kD band detected, being only 164 (CRIP1a) and 128 (CRIP1b) amino acids in size, their existence raises the possibility that other proteins that specifically interact with the cannabinoid receptors may be present in cells. The receptor may also be able to interact with other, non-cannabinoid receptor related proteins. For example, although receptor activity-modifying proteins were initially found to be associated with class II GPCRs it is now becoming apparent that they may associate with all types of GPCRs (Hay *et al.*, 2006).

This 83kD form may also be one of the cannabinoid receptor-like receptors. Given that it is detected by N-terminal antibodies raised against the classical CB_1R it would have to be identical in these regions but that would not prevent it having a different pharmacological profile as ligand binding has been shown to occur in the pore created by the TM domains (Mukhopadhyay *et al.*, 2002, Murphy & Kendall, 2003). Given that it is still not certain what this 83kD protein is, but the data suggests it is a form of the receptor, it will be referred to as a modified CB_1R protein throughout the rest of this study.

3.8.2 CB_2R protein expression in leukaemic T lymphocyte cell lines

The CB_2R is a much smaller protein than the CB_1R and its predicted molecular weights is 39kD based on its amino acid sequence. Using the Cayman

Chemicals CB_2R antibody a band of this approximate size was detected in both Jurkats and CEMs. This was expected as Jurkats have previously been shown to express CB_2R mRNA (Schatz *et al.*, 1997, McKallip *et al.*, 2002a) and CEMs have previously been shown to express low levels of CB_2R by comparing binding of biotinylated Δ^9-THC to cells in the presence and absence of CB_1R and CB_2R antagonists (Powles *et al.*, 2005). Although the CB_2R does have one potential *N*-glycosylation site and a 46kD glycosylated form of the receptor has been detected in other studies (Carayon *et al.*, 1998, Ligresti *et al.*, 2003, Filppula *et al.*, 2004, Wright *et al.*, 2005) only one band was ever detected in the cells used in this study. This suggests that in these cells the receptor may not be *N*-glycosylated or that very low levels are expressed and not detected by the antibody. It is unlikely that the Cayman Chemicals antibody is not able to detect the 46kD form as it has previously done so in other studies (Ligresti *et al.*, 2003, Wright *et al.*, 2005). Thus, Jurkats and CEMs express both the CB_2R and at least one form of the CB_1R, making them suitable for use in the second part of this study which focuses on the role of cannabinoids in T lymphocyte function.

3.8.3 Which cannabinoid receptors are expressed in naïve peripheral T lymphocytes?

Although it has been well established that primary T lymphocytes express CB_2R mRNA (Galiegue *et al.*, 1995), data published concerning CB_2R protein expression is contradictory as already mentioned. It is know that CB_2R mRNA expression in T lymphocytes is low compared to other immune subpopulations (Galiegue *et al.*, 1995), therefore it is possible that these differences may be due to sensitivity of the techniques used in these studies. Using the Cayman Chemicals CB_2R antibody the CB_2R protein was not detected in WCLs of human naïve peripheral T lymphocytes. It is possible that the level of CB_2R protein fell below the detection limit of the antibody or that under basal conditions the mRNA is simply not translated into protein. For example, it was recently published that even though polymorphonuclear cells had been shown to express CB_2R mRNA no functional protein could be detected (Deusch *et al.*, 2003). Only the 83kD, modified, CB_1R was detected in these naïve T lymphocytes.

Similarly, some but not all PBMC populations appeared to express the CB_2R protein although CB_2R mRNA (Nong *et al.*, 2002) and low levels of protein (Gardner *et al.*, 2002) have previously been detected in human PBMCs. Again, it may simply be that in certain instances the level of CB_2R protein fell below the detection limit of the antibody. This could be due to either subtle differences in antibody specificity between batches and/or dilutions of the antibody or donor variation. It is currently not clear whether cannabinoid receptor expression varies among the population. One study has shown that CB_1R expression in humans is dependent on gender and ethnic background although the blots they present are not particularly clear (Onaivi *et al.*, 1999). Receptor mRNA levels have also been shown to differ between individual donors in one study although they did not investigate whether this correlated with any differences in age or gender (Schwarz *et al.*, 1994). Another study has shown that neither age, gender nor ethnic origin affected the expression of CB_1R and CB_2R mRNA in human PBMCs (Nong *et al.*, 2002).

Oddly, the CB_2R was never detected in the PBMC population whilst the effects of SEB-induced activation on receptor expression in T lymphocytes were being investigated, but was detected in samples collected to examine the effects of CD3/CD28-induced activation. Again, this suggests that the difference is likely to be due to differences in batches of antibody as these experiments were carried out at different times. However, it is important to note that the PBMCs were not treated in quite the same manner. The PBMCs used in the SEB-activation experiments are freshly isolated whereas those used in the CD3/CD28-induced activation experiments are monocyte depleted. It is possible that by removing the monocytes from the culture and thereby increasing the density of lymphocytes, this increases the relative concentration of CB_2R protein. Further investigation is needed to verify whether this is possible. For instance, by characterising the PBMC population before and after monocyte depletion both in terms of cannabinoid receptor expression and also in terms of which cells are present in the population. Again, only the modified form of the CB_1R was detected in all of the PBMCs.

3.8.4 What is the effect of activation on cannabinoid receptor expression in T lymphocytes?

CB_2R, but not CB_1R, protein expression was significantly upregulated when the T lymphocytes in the PBMC population were activated using the superantigen SEB and clonally expanded to generate PBLs. This method of generating and culturing PBLs yielded an almost pure T lymphocyte population indicating that the increase in CB_2R protein was not due to contamination from other cell types. The increase in CB_2R protein expression detected was transient as after several days the receptor levels were again downmodulated. Expression peaked around day 5 after initial PBMC isolation. The difference in CB_2R protein expression between day 5 PBLs, when expression was high, and day 12 PBLs, when expression had been downregulated, was also detected using a second CB_2R-specific antibody. That CB_2R expression was modulated following activation was not surprising as previous studies have reported that cannabinoid receptor expression in immune cells can be upregulated or downregulated by a variety of factors (Table 4). Many of these investigations have focused on the changes in receptor mRNA expression over 24 or 48 hours elicited by cellular activation and not the long-term effects on protein expression. However, there are exceptions. For instance, CB_2R mRNA and protein levels were shown to change as B lymphocytes differentiate from virgin to memory B lymphocytes (Carayon *et al.*, 1998) and the CB_2R was found to be differentially expressed in relation to cellular activation of rat peritoneal macrophages at both the mRNA and protein level (Carlisle *et al.*, 2002). The importance of examining not just mRNA but also protein levels is highlighted by a report which showed that expression of CB_2R mRNA but not protein was higher in immature compared to mature murine dendritic cells (Maestroni, 2004).

The results from the current study also raised the question of whether the mode of cellular activation would affect CB_2R protein upregulation. CD3/CD28-coated beads were used to activate the T lymphocytes in the PBMC population. Again, the expression of the 83kD form of the CB_1R remained constant throughout the culture period. However, CB_2R upregulation was maintained for longer than when the T lymphocytes were activated using the superantigen SEB.

SEB acts by binding, as an intact molecule, to the class II MHC complex expressed on professional APCs, such as the B lymphocytes found in the PBMC population, and then sequentially binding the TCR, bringing them together to induce T lymphocyte activation (Proft & Fraser, 2003). Whether co-stimulation of CD28 is required for activation by superantigens remains controversial (Damle *et al.*, 1993, Muraille *et al.*, 1995, Krummel *et al.*, 1996, Rajagopalan *et al.*, 2002). The beads are coated in CD3 and CD28 antibodies and thereby mimic the *in vivo* stimulation of T lymphocytes by APCs by binding not only the CD3 but also inducing co-stimulatory signals by binding CD28. Thus there may be differences in the pathways activated by these two methods of T lymphocyte activation. Unfortunately, to the best of my knowledge, nothing is currently known about what promoter the cannabinoid receptors are under control of and hence it is not possible to speculate how the two methods of cellular activation would act differently in this respect. However, as well as possibly activating the T lymphocytes in slightly different ways there is another key difference in how these two types of PBLs are cultured. Whereas SEB is only added to the cells once, the CD3/CD28-coated beads remain in the tissue culture flasks throughout the culture. It is possible that as the CD3/CD28-activated PBLs are expanding, the beads are again activating the new cells and hence this is why the levels of CB_2R protein expression do not return towards basal as in the SEB-activated PBLs. One way of testing this would be to grow two cultures up from the same source of PBMCs, one which continues to have the CD3/CD28-coated beads in the culture, the other where they are removed on, for example, day 7 and then compare the levels of CB_2R expression.

That the CB_2R but not the CB_1R is upregulated is likely to reflect a difference in promoters for these two receptors. However, this finding was somewhat unexpected as CB_1R mRNA expression has been shown to be regulated in immune cells by a number of mitogens including the T lymphocyte mitogen PHA (Table 4). Nonetheless, in light of these findings it is clear that the role or effect of cannabinoids may change throughout the course of the T lymphocyte response to inflammation and that the circumstances under which the T lymphocytes are activated could influence cannabinoid effects on the cells.

		Stimulus	Cell type	References
CB_1R	↑ mRNA	PHA	Jurkats	Daaka *et al.*, 1996
		Anti-CD40 antibody	mouse splenocytes	Noe *et al.*, 2000
		LPS	mouse macrophages	Klein *et al.*, 2003
		Marijuana use	human PBMCs	Nong *et al.*, 2002
	↑ protein	PHA	Jurkats	Daaka *et al.*, 1996
	↓ mRNA	PHA	human PBMCs	Nong *et al.*, 2002
		Anti-CD3 antibody	mouse splenocytes	Noe *et al.*, 2000
		PMA/Ionomycin	mouse splenocytes	Noe *et al.*, 2000
CB_2R	↑ mRNA	Anti-CD40 antibody	mouse splenocytes	Lee *et al.*, 2001
		Marijuana use	human PBMCs	Nong *et al.*, 2002
		IFNγ	rat peritoneal macrophages	Carlisle *et al.*, 2002
		IFNγ + GM-CSF	mouse microglial cells	Maresz *et al.*, 2005
	↑ protein	Anti-CD3 antibody	human lymphocytes	Gardner *et al.*, 2002
		IFNγ	rat peritoneal macrophages	Carlisle *et al.*, 2002
		SEB	human PBLs	
		CD3/CD28-coated beads	human PBLs	
	↓ mRNA	Differentiation	B lymphocytes	Carayon *et al.*, 1998
		LPS	mouse splenocytes	Lee *et al.*, 2001, McKallip *et al.*, 2002b
		Concanavalin A	mouse splenocytes	McKallip *et al.*, 2002b
	↓ protein	TGFβ	human lymphocytes	Gardner *et al.*, 2002
		Differentiation	B lymphocytes	Carayon *et al.*, 1998

Table 4: **Overview of modulation of cannabinoid receptor expression by cell activation.** CB_1R and CB_2R mRNA expression can be increased or decreased by several different factors. Results from this current study are highlighted.

3.8.5 CB_1R-CB_2R heterodimerisation

Several papers have suggested that the CB_1R can form heterodimers. For instance, specific association of the CB_1R with other receptors such as the LPA1 receptor (Wright *et al.*, 2005), D2 receptor (Kearn *et al.*, 2005), orexin 1 receptor (Hilairet *et al.*, 2003) and possibly opioid receptors (Rios *et al.*, 2006) has been reported. The CB_1R may also exist as homodimers as discussed earlier, as could the CB_2R (Filppula *et al.*, 2004). However, to the best of my knowledge there have been no reports on CB_1R-CB_2R dimerisation. In order to investigate CB_1R-CB_2R association, HT-29s were used as they have previously been reported to express both receptors (Ihenetu *et al.*, 2003b, Wright *et al.*, 2005). Using immunoprecipitation, the CB_2R was detected in anti-CB_1R IP samples from HT-29s. This preliminary data suggests that the CB_1R and CB_2R associate in cells that express both receptors and thus may form heterodimers or even larger oligomers. However, further investigation is needed to corroborate this data. For instance, it must be verified that the CB_1R can be detected in anti-CB_2R IPs, and ideally that the association also occurs in primary cells. In particular, it remains to be seen whether the modified CB_1R detected in T lymphocytes can associate with the CB_2R. Techniques such as fluorescent resonance energy transfer or bioluminescence resonance energy transfer could be used to determine whether the two receptors are closely associated or not. It would also be interesting to study whether agonist binding would affect dimer formation as in other studies (Kearn *et al.*, 2005, Wright *et al.*, 2005).

The implications of CB_1R–CB_2R association are numerous, especially as the CB_2R is being detected in more cell types and hence it is becoming clear that not only immune cells express both receptors. For instance, given that it is well established that GPCR heterodimerisation can alter the pharmacology normally attributed to the individual receptor (Maggio *et al.*, 2005, Prinster *et al.*, 2005), it may account for some of the cannabinoid pharmacology seen in certain cell types that has, up until now, been explained by the existence of additional cannabinoid-like receptors (Wiley & Martin, 2002, Begg *et al.*, 2005). The existence of dimers may also change with cellular activation and/or differentiation as receptor expression can be altered under these circumstances as already discussed.

Equally, dimer formation could also be increased in disease states. For example, primary human colonic epithelial cells only express the CB_1R but the CB_2R can be detected in these cells in sections taken from Crohn's disease patients (Wright *et al.*, 2005). Therefore, under normal physiological conditions cells may only express one type of cannabinoid receptor but under pathological conditions begin to express both. This could greatly affect how the cells respond to cannabinoids not only because of the activation of the individual receptor being upregulated but also because of the activation of dimers.

3.8.6 Do primary T lymphocytes express other proteins of the cannabinoid system?

In addition to expressing both cannabinoid receptors, PBLs also express both FAAH and MAGL, two enzymes involved in endocannabinoid inactivation (Goparaju *et al.*, 1998, Cravatt & Lichtman, 2002, Dinh *et al.*, 2002). FAAH has previously been detected in human T lymphocytes (Maccarrone *et al.*, 2003c). However, although MAGL mRNA has been detected in a wide range of tissues including mouse and rat spleen (Karlsson *et al.*, 1997, Karlsson *et al.*, 2001) and has been purified from several different cell types (Goparaju *et al.*, 1998), to my knowledge there have been no reports looking at its expression in T lymphocytes. Interestingly, FAAH appeared as a doublet and ran at a slightly higher molecular weight in Jurkats and CEMs compared to the primary PBLs. This suggests that in these cell lines FAAH may undergo some post-translational modifications. That the FAAH expressed in cell lines may be different from that in primary cells underlines the need carry out experiments in primary cells, especially if FAAH is being considered as a therapeutic target (Di Marzo *et al.*, 2004). It would also be interesting to investigate whether FAAH and/or MAGL expression is affected by T lymphocyte activation. LPS has been reported to downregulate FAAH gene expression in human peripheral lymphocytes (Maccarrone *et al.*, 2001) and it is also regulated by leptin and progesterone (Maccarrone *et al.*, 2003a, Maccarrone *et al.*, 2003c). Thus it is certainly possible that FAAH protein levels may be altered by cellular activation.

3.9 Conclusions

In this part of the study it was determined that CB_2R protein is upregulated following human T lymphocyte activation. The mode of cellular activation appears to influence how long the CB_2R is upregulated for. One of the main aims of the next part of this study will be to investigate whether this difference in CB_2R expression translates into a difference in cannabinoid-mediated effects on T lymphocyte function. For now, it does suggest that the role of cannabinoids in the T lymphocyte-mediated immune response may change as the T lymphocyte becomes active. Furthermore, as different modes of activation appear to influence cannabinoid receptor expression there is potential for a highly regulated and complex system.

Only an 83kD protein was detected in naïve and activated T lymphocytes using the Cayman Chemicals CB_1R antibody. Data collected from the colonic epithelial cell line HT-29s and two human leukaemic T lymphocyte cell lines, Jurkats and CEMs, suggest it is a modified form of the CB_1R but the nature of this modification has not been identified. It remains unclear whether the modified receptor could be functional or not and this issue will be addressed in the next part of this study. However, it is not *N*-glycosylated. In addition to expressing the CB_2R and a modified CB_1R, activated human T lymphocytes also express both FAAH and MAGL, two enzymes involved in endocannabinoid degradation. Preliminary evidence also suggests that the CB_1R and CB_2R may form heterodimers in cells which express both receptors but this needs to be further investigated.

Chapter 4: Results II – Function of cannabinoid receptors in T lymphocytes

4.1 Background

Cannabinoids have been linked to many different intracellular signalling pathways. These include the activation of the MAPKs, ERK1/2 (Bouaboula *et al.*, 1995b, Bouaboula *et al.*, 1996, Galve-Roperh *et al.*, 2002, Derkinderen *et al.*, 2003, Samson *et al.*, 2003), which is induced by cannabinoids in many different cells types including Jurkats (Herrera *et al.*, 2005, Ghosh *et al.*, 2006). Cannabinoids have also been shown to modulate cAMP production (Pertwee, 1997, Kaminski, 1998, Howlett *et al.*, 2002, Demuth & Molleman, 2006). For example, they inhibit forskolin-stimulated cAMP production in lymphocytes (Diaz *et al.*, 1993, Condie *et al.*, 1996, Mechoulam *et al.*, 1995) as well as cAMP response element (CRE) DNA binding activity, a part of the cAMP signalling cascade downstream of PKA (Koh *et al.*, 1997, Herring & Kaminski, 1999, Kaplan *et al.*, 2005b).

Just as they have been linked to numerous signalling pathways, cannabinoids have also been shown to affect a number of different immune cell functions, such as migration and proliferation. Several studies have suggested that 2-AG acts as a chemoattractant for immune cells including the HL-60 cell line which had been differentiated into macrophage-like cells (Kishimoto *et al.*, 2003), CB_2R-expressing myeloid precursor cells (Jorda *et al.*, 2003), B lymphocytes (Jorda *et al.*, 2002, Rayman *et al.*, 2004) and eosinophils (Oka *et al.*, 2004). However, the response appears to be cell-type and cannabinoid-specific as other cannabinoids, including AEA, have been shown to have no effect on basal cell migration in some studies (Kishimoto *et al.*, 2003, Joseph *et al.*, 2004, Oka *et al.*, 2004).

Cannabinoids have been shown to both enhance and suppress immune cell numbers. For instance, Δ^9-THC has been reported to suppress mitogen-induced proliferation of murine splencoytes *in vitro* (Pross *et al.*, 1990, Schatz *et al.*, 1993, McKallip *et al.*, 2002a) and human T lymphocyte proliferation induced by allogeneic dendritic cells (Yuan *et al.*, 2002). However, some studies have also shown that cannabinoids can increase proliferation of immune cells (Derocq *et al.*, 1995, Derocq *et al.*, 1998, Valk *et al.*, 1997). In particular, one study demonstrated that whilst high concentrations of Δ^9-THC inhibited mitogen-

induced proliferation of human peripheral blood lymphocytes, low concentrations increased it, suggesting that cannabinoid effects on lymphocyte proliferation may be concentration-dependent (Luo *et al.*, 1992).

The main aim of this part of the study was to explore the effects of cannabinoids on migration and proliferation of activated T lymphocytes. In conjunction with this the effects of cannabinoids on ERK phosphorylation and cAMP production were first examined to determine whether cannabinoids activated intracellular signalling pathways downstream of the cannabinoid receptors. Day 5 SEB-activated PBLs were the main cell type used in this section of the study as it was shown in *Results I* that this is when CB_2R expression peaks. Some experiments were also repeated in day 11-12 SEB-activated PBLs in order to investigate whether the decrease in CB_2R protein expression detected was reflected in any changes in functional effects of the cannabinoids. Some experiments were also carried out using the leukaemic T lymphocyte cell line Jurkats in order to corroborate data generated using the PBLs.

4.2 Cannabinoid-induced activation of signalling pathways in T lymphocytes

4.2.1 Effects of AEA, 2-AG and JWH-133 on ERK1/2 phosphorylation in T lymphocytes

Having established, in the first part of this study, that day 5 SEB-activated PBLs express the CB_2R and a modified form of the CB_1R it was necessary to establish whether the cannabinoids coupled to biochemical pathways in these cells, and if so whether the receptors were involved, before investigating the functional effects of cannabinoids in these cells. The rapid phosphorylation and activation of the ERK1/2 MAPKs has been documented to lie downstream of the cannabinoid receptors in several different cell types (Bouaboula *et al.*, 1995b, Bouaboula *et al.*, 1996, Galve-Roperh *et al.*, 2002, Derkinderen *et al.*, 2003, Samson *et al.*, 2003) and thus seemed an appropriate signal to study. In addition, ERK has been reported to be involved in migration (Klemke *et al.*, 1997, Huang *et al.*, 2004) and proliferation (Stork & Schmitt, 2002) and thus this pathway was

chosen as there was the possibility that its involvement in cannabinoid-mediated effects on PBL migration and proliferation could be investigated if any responses were detected. ERK1/2 are activated by the upstream kinase MEK via phosphorylation in the activation loop (Kolch, 2000) and detecting this phosphorylation represents an indirect way of determining ERK1/2 activation. PMA was used as a positive control for ERK1/2 phosphorylation in most of the experiments as it is well established as a robust activator of ERK1/2 (Brose & Rosenmund, 2002, Moelling *et al.*, 2002).

Day 5 and 12 PBLs and Jurkats were stimulated with the non-CB_1R/CB_2R selective agonists AEA and 2-AG. The reported Ki values of AEA for the CB_1R and CB_2R are 89nM and 371nM respectively, revealing a slight preference for CB_1R at lower concentrations (Howlett *et al.*, 2002). Similarly, the Ki values for 2AG are 472nM and 1400nM (Howlett *et al.*, 2002). These two cannabinoids were chosen to be used in this study because of their physiological relevance as endocannabinoids. In addition, 2-AG would later also be used in the functional studies. Stimulation of all three cell types with AEA at concentrations unlikely to discriminate between the CB_1R and CB_2R, resulted in a transient increase in ERK1/2 phosphorylation above basal (Fig. 4.1). This response was concentration-dependent, with maximal phosphorylation elicited by 10μM of AEA. As full concentration response curves were not carried out it is not possible to determine EC_{50} values in each of the cell types but the curves in Fig. 4.1*D* show that AEA appears to be more potent in Jurkats compared to PBLs. 2-AG also elicited concentration-dependent increases in ERK phosphorylation in all three cell types (Fig. 4.2).

A CB_2R-selective synthetic agonist, JWH-133 was also used. The reported Ki is 3.4nM at the human CB_2R expressed in HEK293 cells (compared to 677nM for the CB_1R expressed in rat brain; Howlett *et al.*, 2002). JWH-133 also stimulated ERK1/2 phosphorylation in day 5 and 12 PBLs and Jurkats in a concentration-dependent and transient manner (Fig. 4.3). Again, although EC_{50} values could not be calculated, the curves in Fig. 4.3*D* show that JWH-133 appears to be more potent in Jurkats compared to PBLs. This data suggests that the CB_2R expressed in day 5 PBLs is indeed active and coupled to downstream effectors. That all

three cannabinoids tested induced concentration-dependent increases in ERK1/2 phosphorylation in the day 12 PBLs (Fig. 4.1*B*, 4.2*B* and 4.3*B*), when CB_2R expression is severly downregulated but the modified CB_1R is still expressed, suggests that either enough CB_2R is still expressed to signal to downstream effectors or that the modified CB_1R is a functional receptor.

The ability of AEA and 2-AG to induce ERK1/2 phosphorylation in naïve human T lymhocytes was also briefly investigated. In the first part of this study no CB_2R protein and only the modified form of the CB_1R was detected in these cells. Preliminary evidence suggests that neither AEA nor 2-AG stimulated ERK1/2 phosphorylation, even at 10μM (Fig. 4.4). However, PMA stimulated a robust and easily detectable increase in levels of phosphorylated ERK1/2 (Fig. 4.4). Another kinase which has been shown to be activated downstream of the cannabinoid receptors is PKB (Gomez *et al.*, 2000, Galve-Roperh *et al.*, 2002, Molina-Holgado *et al.*, 2002, Sanchez *et al.*, 2003). Phosphorylation at Ser473 is an important step in PKB activation and thus can be used as an indirect measure of its activation status (Vanhaesebroeck & Alessi, 2000, Song *et al.*, 2005). Phosphorylation was not stimulated in the naïve T lymphocytes by either AEA or 2-AG (Fig. 4.4). To verify that this phosphorylated form of PKB could be detected by immunoblotting a sample of unstimulated Jurkats, which are known to constitutively express high levels of phosphorylated PKB (Astoul *et al.*, 2001), were also resolved on the gel and were detected. These results suggest that the modified CB_1R expressed in these cells may not be functional.

AEA and 2-AG are normally rapidly taken up by cells and inactivated. Several enzymes can degrade the endocannabinoids. For example, FAAH hydrolyses both AEA and 2-AG and MAGL metabolises 2-AG (Goparaju *et al.*, 1998, Cravatt & Lichtman, 2002, Dinh *et al.*, 2002). Both of these enzymes were shown to be expressed in PBLs and Jurkats in *Results I.* Therefore, it was investigated whether degradation of AEA and 2-AG affects their ability to stimulate ERK1/2 phosphorylation. The potent FAAH and MAGL inhibitor MAFP, which has a reported IC_{50} of 1-3nM, was used for this (De Petrocellis *et al.*, 1997, Goparaju *et al.*, 1999, Saario *et al.*, 2004).

Preliminary evidence from experiments carried out on day 12 PBLs and Jurkats (Fig. 4.5) showed that pre-treating cells with MAFP (1μM) nearly abolished the increase in ERK1/2 phosphorylation induced by various concentrations of AEA and 2-AG completely. This suggested that the responses detected were actually due to AEA and 2-AG metabolites, rather than the endocannabinoids themselves, as preventing this degradation attenuated the responses. These experiments were then repeated using day 5 PBLs as these cells were used for the majority of other experiments. MAFP treatment of the cells alone caused a slight increase in ERK1/2 phosphorylation at 2 minutes but had no effect by 10 minutes, the length of time of pre-treatment (Fig. 4.6*A*). A decrease in ERK1/2 phosphorylation was detected when day 5 PBLs were pre-treated with MAFP (1μM) and then stimulated with AEA (Fig. 4.6*B*). This was consistent with the effects detected in day 12 PBLs and Jurkats. However, this response was not concentration-dependent and was mimicked when cells were pre-treated with the vehicle equivalent of MAFP (ethanol, final concentration 0.076%; Fig. 4.6*C*). Furthermore, MAFP also affected the ERK1/2 phosphorylation stimulated by JWH-133, a synthetic, stable cannabinoid (Fig. 4.5*C*). This data indicates that the decrease in ERK1/2 phosphorylation is not due to pre-treatment of the cells with MAFP and suggests that the responses are in fact mediated by 2-AG and AEA rather than breakdown products.

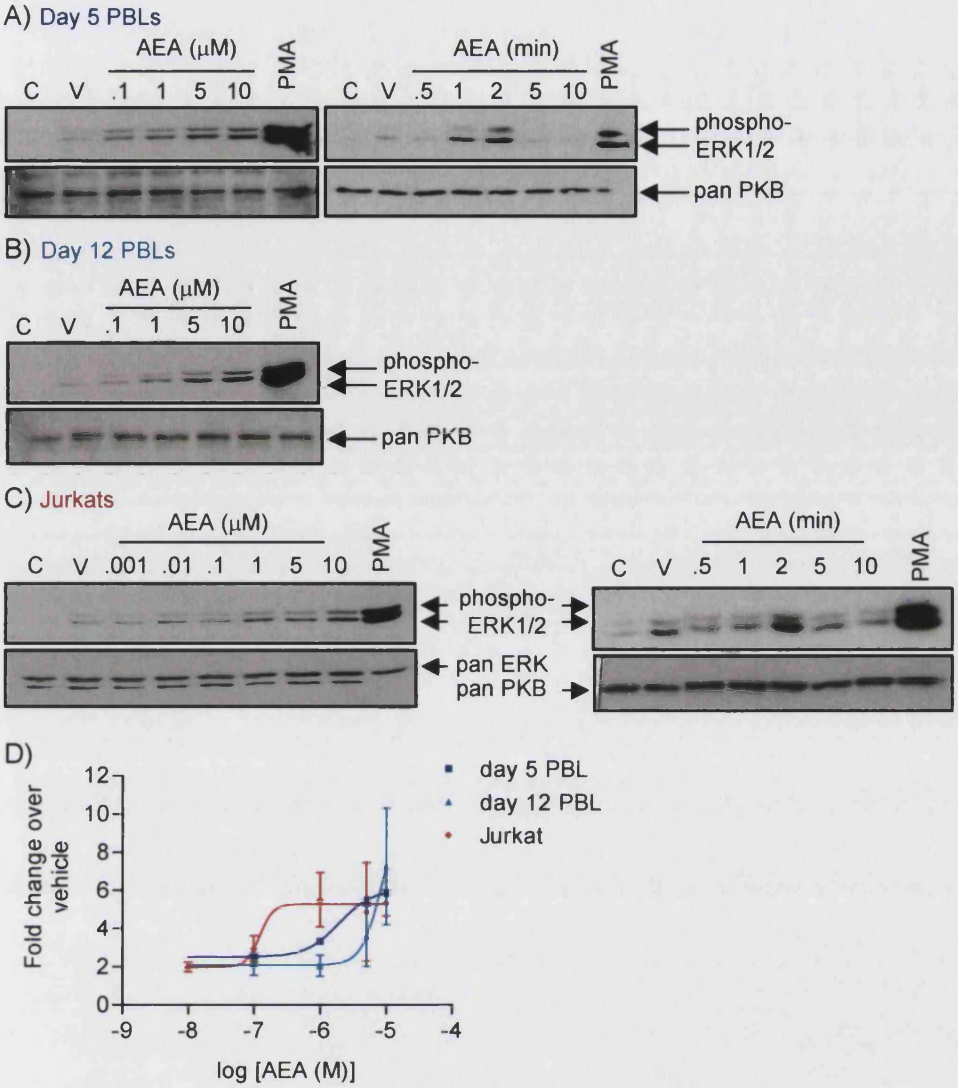


Figure 4.1: **AEA stimulates phosphorylation of ERK1/2 in PBLs and Jurkats.** Day 5 (*A*) and day 12 (*B*) SEB-activated PBLs and Jurkats (*C*) were stimulated with various concentrations of AEA (as indicated) for 2 minutes or 10μM AEA for various times (as indicated). Cells were subsequently lysed as described in *Materials and Methods* and WCLs ($3.2x10^6$ cells per lane for PBLs, $1.6x10^6$ cells per lane for Jurkats) resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK, and protein was visualised with ECL. The blots were stripped and reprobed with pan ERK or pan PKB antibody to verify equal loading and efficiency of protein transfer. Vehicle controls were also carried out (V, 2 minute stimulation with ethanol equivalent to 10μM AEA). Unstimulated cells (control, C) were also lysed to ascertain basal levels of ERK phosphorylation and PMA (100nM, 5 min) was used a positive control for ERK stimulation. Data are representative of 3 separate experiments. *D*) Optical densitometry was carried out on the blots and values expressed as fold change over vehicle. Data are depicted as mean ±S.E.M and are derived from 3 individual experiments.

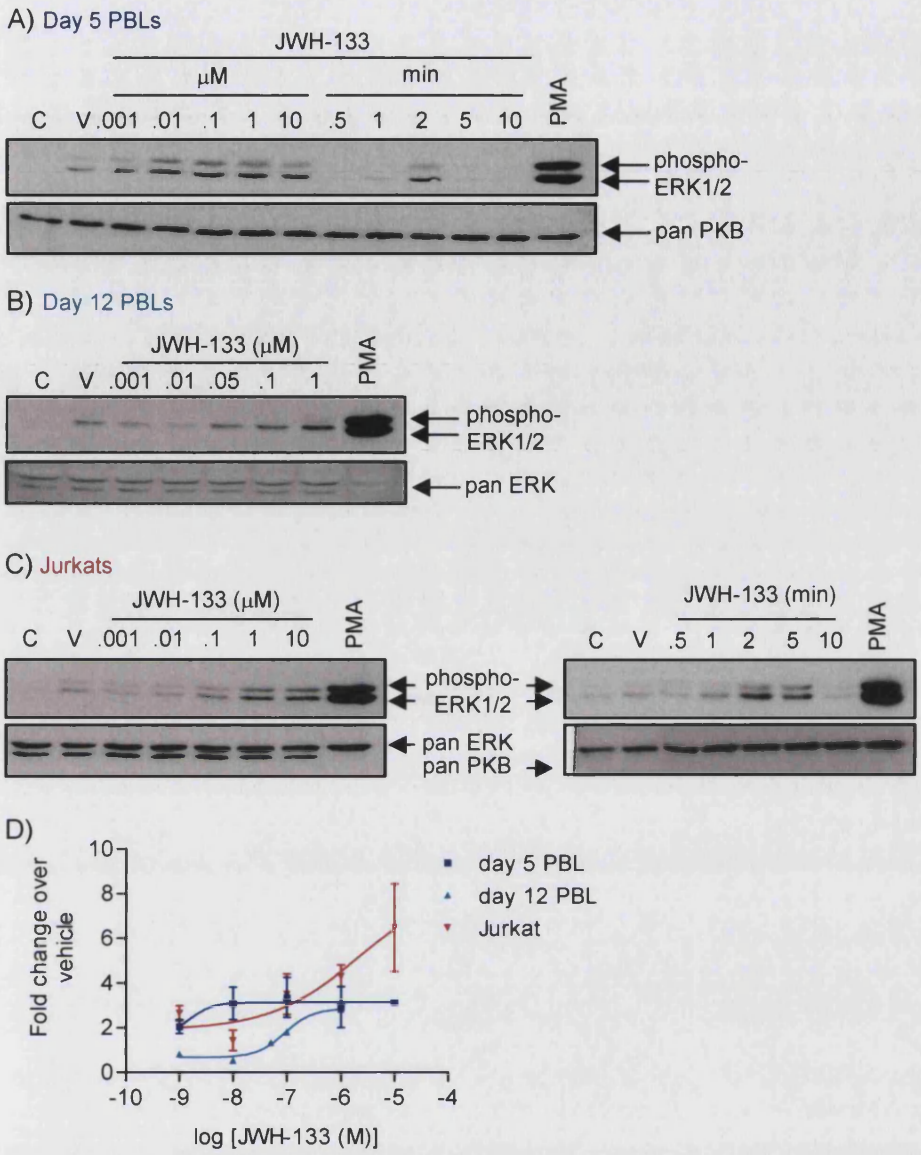


Figure 4.2: **2-AG stimulates phosphorylation of ERK1/2 in PBLs and Jurkats.** Day 5 (*A*) and day 12 (*B*) SEB-activated PBLs and Jurkats (*C*) were stimulated with various concentrations of 2-AG (as indicated) for 2 minutes or 10μM 2-AG for various times (as indicated). Cells were subsequently lysed as described in *Materials and Methods* and WCLs ($3.2x10^6$ cells per lane for PBLs, $1.6x10^6$ cells per lane for Jurkats) resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK, and protein was visualised with ECL. The blots were stripped and reprobed with pan ERK or pan PKB antibody to verify equal loading and efficiency of protein transfer. Vehicle controls were also carried out (V, 2 minute stimulation with ethanol equivalent to 10μM 2-AG). Unstimulated cells (control, C) were also lysed to ascertain basal levels of ERK phosphorylation and PMA (100nM, 5 min) was used a positive control for ERK stimulation. Data are representative of 3 separate experiments. *D*) Optical densitometry was carried out on the blots and values expressed as fold change over vehicle. Data are depicted as mean ±S.E.M and are derived from 3 individual experiments.

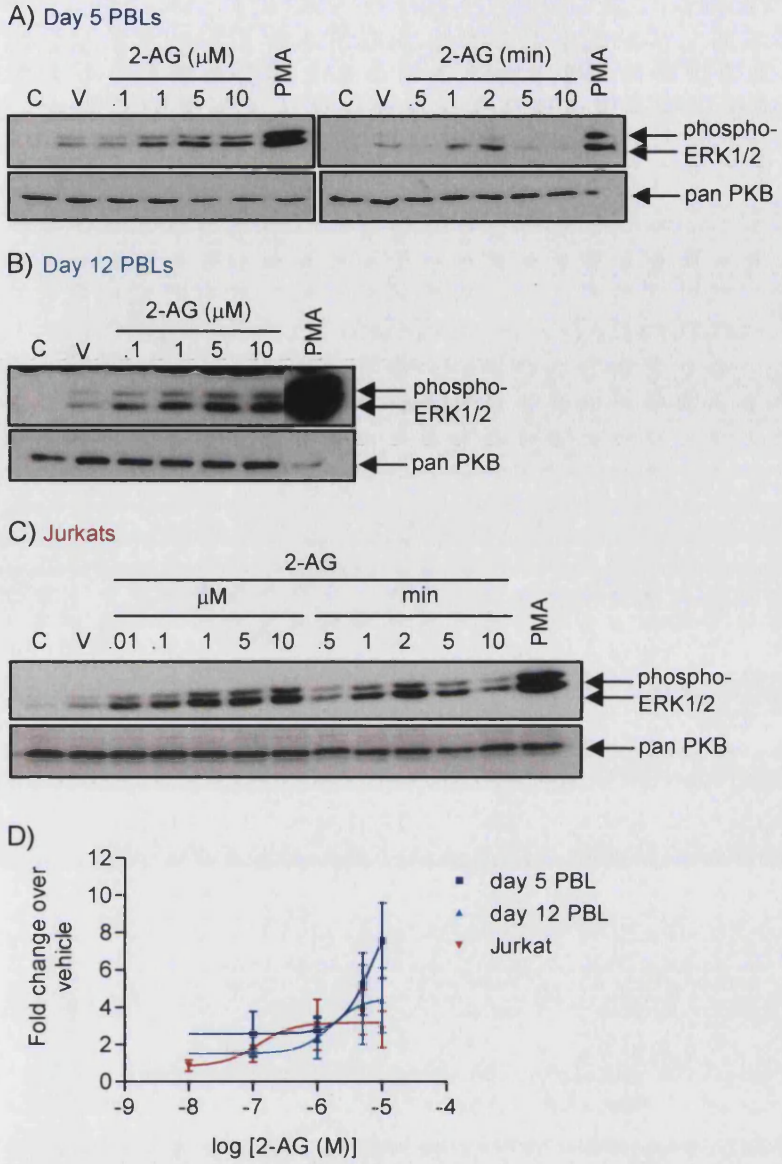


Figure 4.3: **JWH-133 stimulates phosphorylation of ERK1/2 in PBLs and Jurkats.** Day 5 (*A*) and day 12 (*B*) SEB-activated PBLs and Jurkats (*C*) were stimulated with various concentrations of JWH-133 (as indicated) for 2 minutes or 100nM JWH-133 for various times (as indicated). Cells were subsequently lysed as described in *Materials and Methods* and WCLs ($3.2x10^6$ cells per lane for PBLs, $1.6x10^6$ cells per lane for Jurkats) resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK, and protein was visualised with ECL. The blots were stripped and reprobed with pan ERK or pan PKB antibody to verify equal loading and efficiency of protein transfer. Vehicle controls were also carried out (V, 2 minute stimulation with ethanol equivalent to 100nM JWH-133). Unstimulated cells (control, C) were also lysed to ascertain basal levels of ERK phosphorylation and PMA (100nM, 5 min) was used a positive control for ERK stimulation. Data are representative of 2 (*A*), 1 (*B*) or 3 (*C*) separate experiments. *D*) Optical densitometry was carried out on the blots and values expressed as fold change over vehicle. Data are depicted as mean ±S.E.M and are derived from 2 (day 5 PBLs), 1 (day 12 PBLs) or 3 (Jurkats) individual experiments.

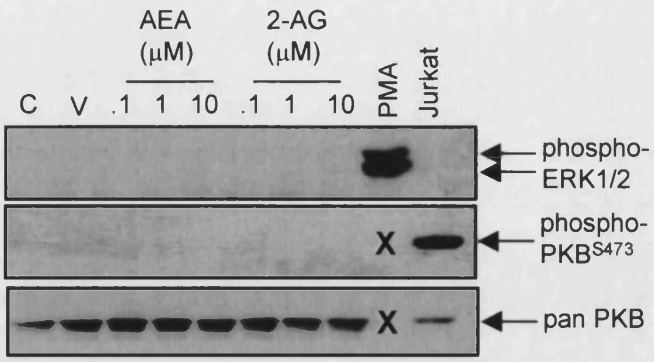


Figure 4.4: **AEA and 2-AG do not stimulate ERK1/2 or PKB phosphorylation in naïve human T lymphocytes.** Naïve T lymphocytes were isolated and purified as described in *Materials and Methods*. Cells were stimulated with 0.1-10μM AEA or 2-AG or vehicle (V, ethanol equivalent to 10μM AEA/2-AG) for 2 minutes and lysed. WCLs ($3.2x10^6$ cells per lane) were resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK or a phospho-specific PKB antibody with affinity for the Ser473 phosphorylated form of PKB, and visualised with ECL. One of the blots was stripped and reprobed with pan PKB antibody to verify equal loading and efficiency of protein transfer. Unstimulated cells (control, C) were also lysed to ascertain basal levels of phosphorylated ERK and PKB and PMA (100nM, 5 min) was used as a positive control for ERK stimulation. A Jurkat WCL ($1.6x10^6$cells) was loaded as a positive control for phospho-PKB blotting. X indicates empty lanes. Data is representative of a single experiment.

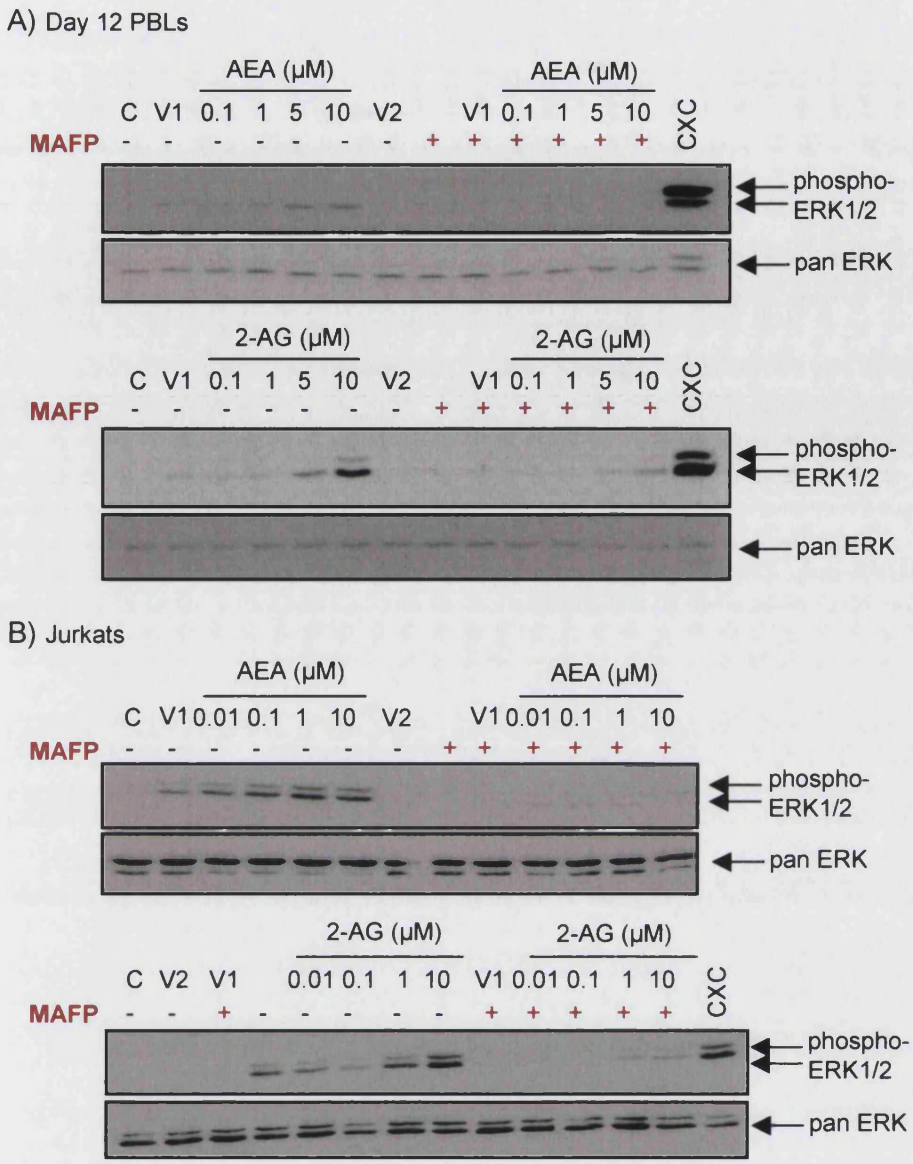


Figure 4.5: **Preliminary evidence suggesting that the ERK1/2 responses induced by AEA and 2-AG are sensitive to MAFP pre-treatment.** Day 12 SEB-activated PBLs (*A*) and Jurkats (*B*) with and without MAFP pre-treatment (1μM, 10 minutes) were stimulated for 2 minutes with AEA or 2-AG (0.1-10μM) and lysed as described in *Materials and Methods*. WCLs ($3.2x10^6$ cells per lane for PBLs, $1.6x10^6$ cells per lane for Jurkats) were resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active, Thr202/Tyr204-phosphorylated form of ERK, and protein visualised with ECL. The blots were stripped and reprobed with a pan ERK antibody to verify equal loading and efficiency of protein transfer. Vehicle controls were also carried out (V1= ethanol equivalent to 10μM AEA for 2 minutes, V2= ethanol equivalent of 1μM MAFP for 10 minutes) and CXCL12 (CXC, 10nM, 2 minutes) was used as a positive control for ERK phosphorylation. Data are representative of 2 separate experiments.

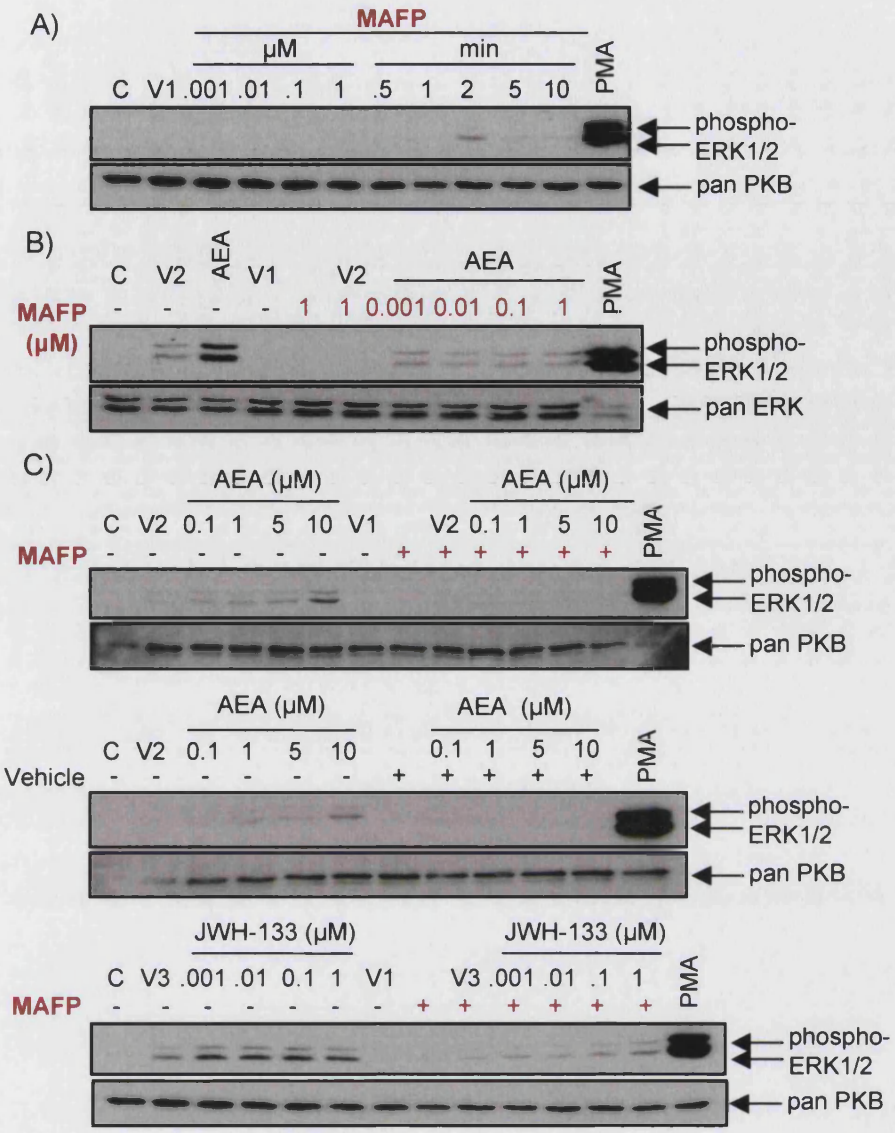


Figure 4.6: **ERK1/2 responses to AEA in day 5 PBLs are insensitive to MAFP pre-treatment.** Day 5 SEB-activated PBLs were treated as follows: *A*) cells were stimulated with MAFP for 10 minutes (0.001-1μM) or with 1μM for various times as indicated. *B*) cells with and without MAFP pre-treatment (0.001-1μM, 10 minutes) were stimulated with 10μM AEA for 2 minutes. *C*) cells were pre-treated either with 1μM MAFP (top blot) or the equivalent concentration of ethanol (vehicle, bottom blot) for 10 minutes and then stimulated with various concentrations of AEA or JWH-133 for 2 minutes. Cells were then lysed as described in *Materials and Methods* and WCLs ($3.2x10^6$ cells per lane) were resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK, and protein visualised with ECL. The blots were stripped and reprobed with either pan PKB or ERK antibody to verify equal loading and efficiency of protein transfer. Vehicle controls were also carried out (V1= ethanol equivalent 1μM MAFP for 10 minutes, V2= ethanol equivalent to 10μM AEA for 2 minutes, V3= ethanol equivalent to 100nM JWH-133 for 2 minutes). Unstimulated cells (control, C) were also lysed to ascertain basal levels of ERK phosphorylation and PMA (100nM, 5 min) was used as a positive control for ERK stimulation. Data are representative of 3 separate experiments.

4.2.2 The role of the cannabinoid receptors in cannabinoid-induced ERK1/2 phosphorylation

Cannabinoid receptor-dependent, as well as independent ERK activation by cannabinoids has been described (Bouaboula *et al.*, 1995b, Derocq *et al.*, 1998, Liu *et al.*, 2000, Berdyshev *et al.*, 2001, Galve-Roperh *et al.*, 2002), therefore it was important to verify whether the ERK1/2 phosphorylation induced by the cannabinoids in this study was receptor mediated. Both of the cannabinoid receptors are GPCRs that couple to Gi/o-proteins (Matsuda *et al.*, 1990, Bayewitch *et al.*, 1995). The Gαi/o-protein inhibitor pertussis toxin (PTX) was used to examine the role of the receptors in signalling. AEA and 2-AG-induced ERK1/2 phosphorylation in day 5 and day 12 PBLs was completely abrogated by PTX pre-treatment (Fig. 4.7*A* and *B*), consistent with a role for the receptors in this response. However, in Jurkats, PTX pre-treatment did not completely abolish the ERK1/2 response (Fig. 4.7*C*), suggesting that in Jurkats there may be an additional receptor-independent component. It was also confirmed, using 5 PBLs, that PTX pre-treatment did not affect receptor protein expression (Fig. 4.7*D*). CXCL12-induced ERK1/2 phosphorylation has previously been shown to be PTX-sensitive (Sotsios *et al.*, 1999) and hence this chemokine was used to verify that PTX was active.

As day 5 SEB-activated PBLs express not only the CB_2R but also an 83kD form of the CB_1R, which may or may not be functional, two receptor antagonists were used to investigate the role of each receptor in cannabinoid-induced ERK1/2 phosphorylation. AM251 is a CB_1R-selective antagonist, with a reported Ki of 7.5nM and 2290nM at the CB_1R and CB_2R, respectively (Palmer *et al.*, 2002). AM630 is a CB_2R-selective antagonist with a reported Ki of 31.2nM at the CB_2R (compared to 5152nM for the CB_1R; Palmer *et al.*, 2002). Day 5 PBLs were pre-treated with the inhibitors prior being stimulated with AEA, 2-AG or JWH-133. AM251 inhibited the AEA and JWH-133 responses, as did vehicle control (0.00008% ethanol; Fig. 4.8). The 2-AG response was less affected by AM251 pre-treatment but again vehicle control also inhibited the response. Likewise, although AM630 inhibited AEA, 2-AG and JWH-133-induced ERK1/2

phosphorylation, this effect was mimicked by the vehicle control (0.004% DMSO). At this time it is unclear why the vehicle controls had these effects but it means that conclusions about which receptor is activated by these cannabinoids cannot be drawn from these experiments.

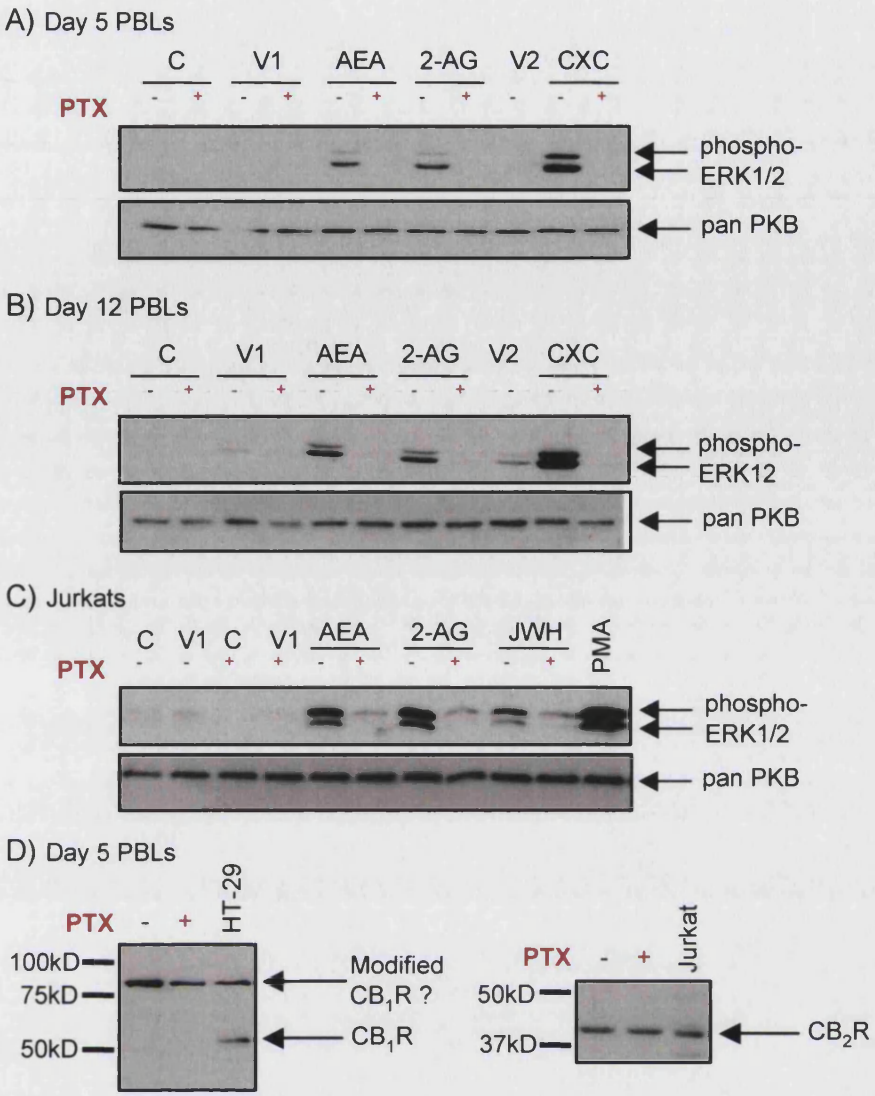


Figure 4.7: **AEA and 2-AG-induced ERK1/2 phosphorylation is PTX-sensitive.** Day 5 (*A*, *D*) and day 12 (*B*) SEB-activated PBLs and Jurkats (*C*) were incubated for 16 hours with 100ng/ml of PTX (+) or 50% glycerol as a control (-) at 37°C in an atmosphere of 5% CO_2. *A-C*) cells were then stimulated with either AEA (10μM), 2-AG (10μM) or JWH-133 (JWH, 100nM) for 2 minutes and subsequently lysed (*A-C*) as described in *Materials and Methods* and WCLs ($3.2x10^6$ cells per lane for PBLs, $1.6x10^6$ cells per lane for Jurkats) resolved by SDS-PAGE gel, transferred to nitrocellulose membranes and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK, and protein was visualised with ECL. The blots were stripped and reprobed with pan PKB antibody to verify equal loading and efficiency of protein transfer. Vehicle controls were also carried out by stimulating cells with ethanol equivalent to 10μM AEA/2-AG (V1). CXCL12 (CXC) a chemokine that also couples to ERK via Gi/o proteins was used to verify that PTX was active (V2=vehicle control for CXCL12) in (*A*) and (*B*). Unstimulated cells (control, C) with and without PTX pre-treatment were also lysed to verify the effect of PTX on basal ERK phosphorylation and PMA (100nM, 5 min) was used as a positive control for ERK phosphorylation in (*C*). *D*) cells were lysed and Cayman Chemicals antibodies were used to verify that PTX pre-treatment had no effect on receptor expression. HT-29 and Jurkat WCLs were used as positive controls for CB_1R and CB_2R expression respectively. Data are representative of three separate experiments.

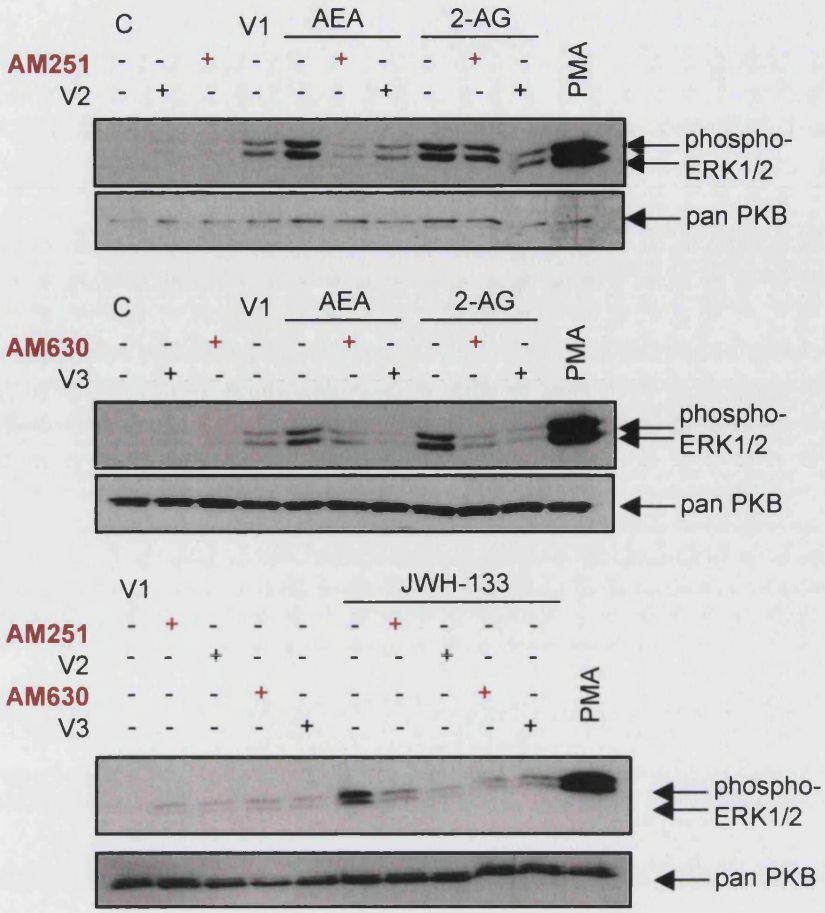


Figure 4.8: **The effects of the inhibitors AM251 and AM630 on cannabinoid-induced ERK1/2 phosphorylation are mimicked by vehicle controls.** Day 5 SEB-activated PBLs with and without AM251 (10nM) or AM630 (50nM) pre-treatment (10 minutes) were stimulated for 2 minutes with AEA (10μM), 2-AG (10μM) or JWH-133 (100nM) and subsequently lysed as described in *Materials and Methods*. WCLs ($3.2x10^6$ cells per lane for PBLs, $1.6x10^6$ cells per lane for Jurkats) were resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active, Thr202/Tyr204-phosphorylated form of ERK, and protein visualised with ECL. The blots were stripped and reprobed with a pan PKB antibody to verify equal loading and efficiency of protein transfer. Vehicle controls were also carried out (V1= ethanol equivalent to cannabinoid, V2= ethanol equivalent of 10nM AM251 for 10 minutes, V3= DMSO equivalent of 50nM AM630 for 10 minutes) and PMA (100nM, 5 minutes) was used as a positive control for ERK phosphorylation. Data are representative of three separate experiments.

4.2.3 Pathways coupling cannabinoids to ERK1/2 phosphorylation in T lymphocytes

A number of pathways have been described to link GPCRs to ERK activation (Marinissen & Gutkind, 2001, Werry *et al.*, 2005) and several proteins have been shown to specifically link cannabinoids to ERK activation (Bouaboula *et al.*, 1996, Sanchez *et al.*, 1998, Liu *et al.*, 2000, Galve-Roperh *et al.*, 2002, Davis *et al.*, 2003, Derkinderen *et al.*, 2003, Samson *et al.*, 2003) as discussed in the *Introduction.* Therefore, a selection of inhibitors was used to determine some of kinases that lie upstream of cannabinoid-induced ERK1/2 phosphorylation in day 5 PBLs. MEK is the dual specificity kinase that activates ERK1/2 via phosphorylation of a Thr-Glu-Tyr-motif in the activation loop (Kolch, 2000). The MEK1 inhibitor PD98059 (Alessi *et al.*, 1995) was used and attenuated AEA-induced increases in ERK1/2 phosphorylation in a concentration-dependent manner in day 5 PBLs (Fig. 4.9*A*). ERK1/2 phosphorylation was almost completely abolished at 20μM PD98059. The PD98059 compound was also tested on CXCL12-induced ERK1/2 phosphorylation to verify that it was active (Fig. 4.9*A*). Thus, AEA-induced ERK1/2 phosphorylation is MEK-dependent in day 5 PBLs. Similarly AEA, 2-AG and JWH-133-dependent ERK1/2 phosphorylation in Jurkats was also abolished by PD98059 pre-treatment (Fig. 4.10).

PKC is one of the many signalling molecules that has been shown to couple GPCRs to ERK1/2 activation. It has also been reported to lie upstream of cannabinoid-induced ERK phosphorylation in CB_2R-transfected CHO cells and ECV304 human umbilical vein endothelial cells (Bouaboula *et al.*, 1996, Liu *et al.*, 2000). In the current study several PKC inhibitors were used to investigate whether PKC lies upstream of AEA-induced ERK phosphorylation in day 5 PBLs. Ro-32-0432 displays some selectivity for certain isoforms at nanomolar concentrations but at the micromolar concentrations used here serves as a general PKC inhibitor (Wilkinson *et al.*, 1993; for details on the IC_{50}s of all the PKC inhibitors used at the individual isoforms please refer to *Appendices*). AEA-induced ERK1/2 phosphorylation was completely abolished by 30μM Ro-32-

0432 (Fig. 4.9*B*), indicating that the response is PKC-dependent. ERK1/2 phosphorylation was also inhibited by the PKCδ inhibitor rottlerin (Gschwendt *et al.*, 1994), but not the PKCα and βI selective inhibitor Gö6976 (Martiny-Baron *et al.*, 1993), suggesting that AEA selectively activates certain PKC isoforms (Fig. 4.9*C*).

The role of PI3K in cannabinoid-induced ERK1/2 phosphorylation in Jurkats was also examined using the non-selective PI3K inhibitor LY294002 (Ward *et al.*, 2003) as PI3K has been shown to be involved in cannabinoid-induced ERK activation in some studies (Bouaboula *et al.*, 1997, Galve-Roperh *et al.*, 2002, Samson *et al.*, 2003, Sanchez *et al.*, 2003). At 10μM LY294002 completely prevented AEA, but not 2-AG, induced ERK1/2 phosphorylation (Fig. 4.10), indicating that this response is at least partly PI3K-dependent in Jurkats but that there may be agonist-specific differences.

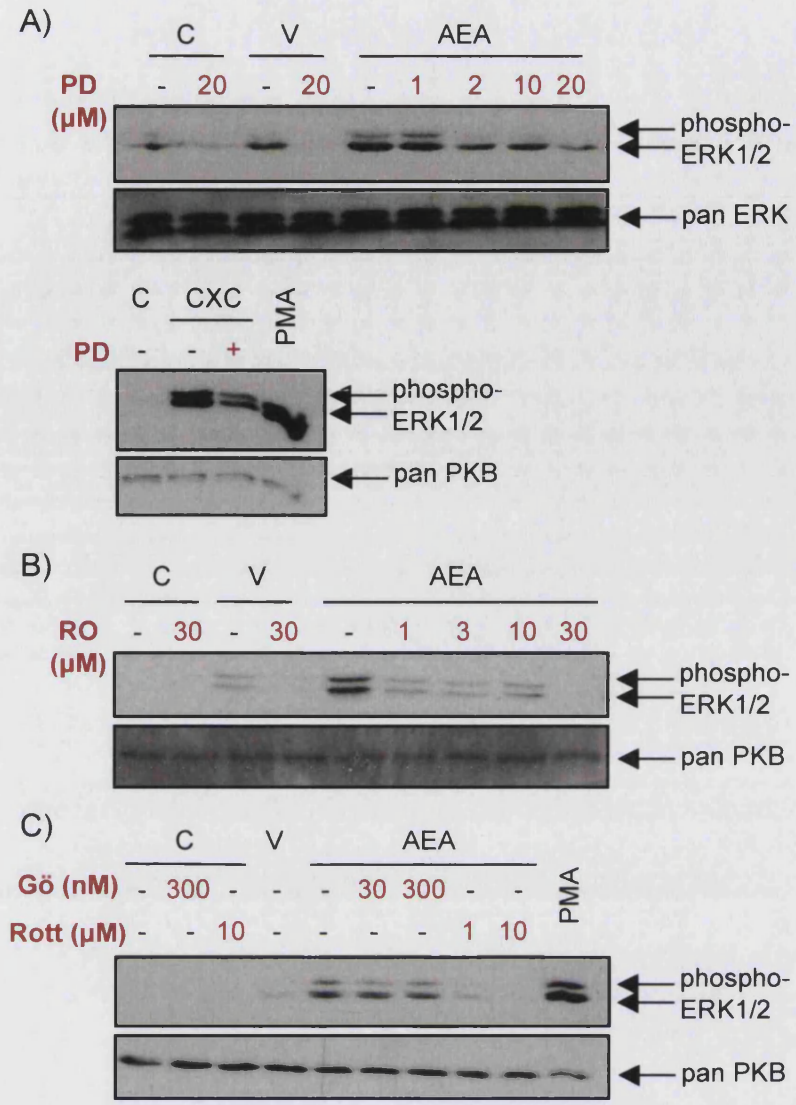


Figure 4.9: **AEA-induced ERK1/2 phosphorylation in day 5 PBLs is MEK and PKC-dependent.** Day 5 SEB-activated PBLs were pre-treated with either PD98059 (PD, *A*) for 1 hour, Ro-32-0432 (RO, *B*) for 10 minutes, Gö6976 (Gö, *C*) for 30 minutes or Rottlerin (Rott, *C*) for 30 minutes at the various concentrations indicated and then stimulated with AEA (10μM), vehicle (V, ethanol equivalent to 10μM AEA) or CXCL12 (CXC, 10nM) for 2 minutes. Samples were subsequently lysed as described in *Materials and Methods* and WCLs ($3.2x10^6$ cells per lane) resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK, and protein was visualised with ECL. The blots were stripped and reprobed with pan PKB or pan ERK antibody to verify equal loading and efficiency of protein transfer. Unstimulated cells (control, C) were also lysed to ascertain basal levels of ERK phosphorylation. In certain cases PMA (100nM, 5 min) was used a positive control for ERK stimulation. Data are representative of three separate experiments.

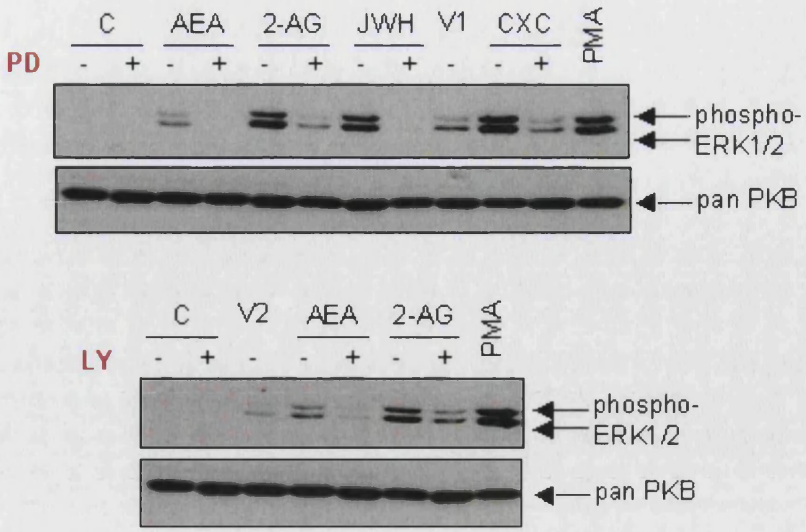


Figure 4.10: **Cannabinoid stimulated ERK1/2 phosphorylation in Jurkats is MEK and PI3K-dependent.** Jurkats were pre-treated with either PD98059 (PD, 20μM) for 1 hour or LY294002 (LY, 10μM) for 30 minutes and then stimulated with AEA (10μM), 2-AG (10μM) or JWH-133 (100nM) for 2 minutes. Samples were then subsequently lysed as described in *Materials and Methods* and WCLs ($3.2x10^6$ cells per lane) resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK, and protein was visualised with ECL. The blots were stripped and reprobed with pan PKB or pan ERK antibody to verify equal loading and efficiency of protein transfer. Unstimulated cells (control, C) were also lysed to ascertain basal levels of ERK phosphorylation and PMA (100nM, 5 min) was used a positive control for ERK stimulation. CXCL12 (CXC, 10nM) was used to verify that PD98059 was active. Vehicle controls were also carried out (V1= vehicle control for CXCL12; V2= ethanol equivalent to 10μM AEA/2-AG). Data are representative of three separate experiments.

4.2.4 Modulation of cAMP pathways by cannabinoids in PBLs

As well as activating ERK, cannabinoids have been shown to affect a number of other signalling pathways, including the production of cAMP. Generally cannabinoids suppress forskolin-induced cAMP production (Pertwee, 1997, Kaminski, 1998, Howlett *et al.*, 2002, Demuth & Molleman, 2006) but they have also been shown to increase forskolin-induced (Glass & Felder, 1997, Felder *et al.*, 1998) and basal (Maneuf & Brotchie, 1997, Steffens *et al.*, 2005a) cAMP production. Preliminary data shows that neither JWH-133 nor 2-AG affected basal production of cAMP in day 5 SEB-activated PBLs, although forskolin, a known AC stimulator (Sunahara & Taussig, 2002), induced a robust increase in intracellular cAMP levels (Fig. 4.11*A*). However, 2-AG significantly increased forskolin-induced cAMP production (Fig. 4.11*B*). The CB_2R-selective agonist, JWH-133, did not mimic this effect (Fig. 4.11*B*).

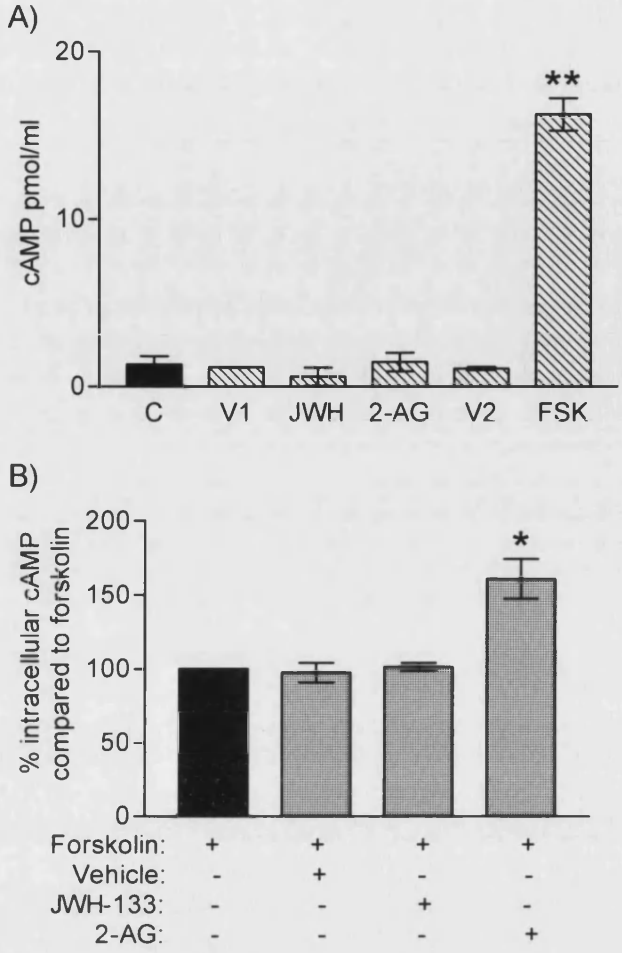


Figure 4.11: **2-AG modulates the production of cAMP in day 5 PBLs.** Day 5 SEB-activated PBLs ($2x10^6$/sample) were treated as follows: *A*) with JWH-133 (JWH, 100nM), 2-AG (10µM) or vehicle (V1, ethanol equivalent to 10µM 2-AG) for 10 minutes or forskolin (FSK, 25µM) or vehicle (V2, ethanol equivalent to 25µM forskolin) for 15 minutes *B*) with JWH-133 (100nM), 2-AG (10µM) or vehicle (ethanol equivalent to 10µM 2-AG) for 10 minutes and then stimulated with forskolin (25µM) for 15 minutes. Cells were then lysed in 0.1N HCl/0.2% triton and intracellular cAMP levels were determined using a cAMP ELISA kit as described in *Materials and Methods*. Data are depicted as mean ±range and are derived from a single experiment (n=2). Data was analysed by ANOVA with Bonferroni correction (*, $p<0.05$ compared to forskolin; **, $p<0.001$ compared to control).

4.3 Cannabinoid-induced modulation of T lymphocyte migration

4.3.1 2-AG and JWH-133 do not induce chemotaxis in T lymphocytes

One of the functions of cannabinoids in the immune system is to modulate cell migration. For instance, during the course of this study, it was published that although AEA did not act as a chemoattractant for naïve $CD8^+$ T lymphocytes, it inhibited their migration to the chemokine CXCL12 and this was suggested to be a CB_2R-mediated effect (Joseph *et al.*, 2004). However, many of the studies on migration were carried in rat or murine cells or transformed cell lines and may not accurately represent what happens in normal human cells. Therefore, day 5 SEB-activated PBLs were used to explore the effect of 2-AG on activated T lymphocytes. The effects of 2-AG on migration of day 12 cells was also studied in order to determine whether they respond differently given that CB_2R expression is reduced compared to day 5 PBLs. A modified Boyden chamber system was used to investigate chemotaxis. In this assay, the ligand in question, for instance 2-AG, is placed in the lower chamber of the apparatus and a membrane placed on top. The cells are then placed in wells in the upper chamber of the apparatus and allowed to migrate. At the end of the assay the number of cells that have migrated are determined.

Before examining whether cannabinoids act as chemoattractants for activated T lymphocytes it was necessary to establish that the PBLs are capable of responding to chemotactic stimuli. The stimulus chosen as a positive control was CXCL12. CXCL12 is a chemokine that acts through the CXCR4 receptor, although it has recently also been shown to bind the orphan receptor RDC1 (Balabanian *et al.*, 2005). It has been well established to induce chemotaxis of T lymphocytes (Sotsios *et al.*, 1999) and both CXCR4 and RDC1 have been shown to be involved (Balabanian *et al.*, 2005). The ability of CXCL12 to couple to intracellular signalling pathways was first verified to establish that it was active. CXCL12 induced both a time and concentration-dependent increase in ERK1/2 and PKB^{Ser473} phosphorylation in day 5 and 12 SEB-activated PBLs. CXCL12

also induced robust, concentration-dependent, chemotaxis of day 5 and day 12 PBLs (Fig. 4.12) as measured using the chemotactic index. This is the ratio of cells migrating towards the ligand versus cells randomly migrating across the membrane. An index of 1 indicates basal migration.

Although the cells were capable of migrating, as indicated by their ability to chemotax in response to CXCL12, 2-AG did not induce chemotaxis in day 5 or day 12 PBLs (Fig. 4.13). The CB_2R-selective agonist, JWH-133 was also tested. Like 2-AG, JWH-133 did not induce migration of either day 5 or day 12 PBLs (Fig. 4.13). These results were somewhat unexpected as 2-AG has been shown to induce migration in several different cell types (Jorda *et al.*, 2002, Jorda *et al.*, 2003, Kishimoto *et al.*, 2003, Oka *et al.*, 2004, Rayman *et al.*, 2004). Therefore, these experiments were repeated in Jurkats and CEMs in order to investigate whether the inability of 2-AG and JWH-133 to elicit chemotaxis was specific to primary T lymphocytes. Neither 2-AG nor JWH-133 induced chemotaxis in Jurkats or CEMs (Fig. 4.14). Again CXCL12 was used as a positive control to verify that the cells were able to migrate and it indeed stimulated robust chemotaxis (Fig. 4.14). Notably, there was some difference in the extent of chemotaxis elicited by CXCL12 in the two sets of Jurkat experiments (Fig. 4.14*A*). This is likely to reflect two different batches of Jurkats being used at different times in the study which respond slightly differently to the CXCL12. This data suggests that 2-AG does not induce migration of T lymphocytes, contrary to data in other cell types.

One of the key steps in cell migration is the polymerisation of actin (Ridley *et al.*, 2003). One way to measure the production of polymerised, F-actin, in cells is to label it with fluorescently tagged phalloidin. This assay involves labelling stimulated, permeabilised cells with FITC-conjugated phalloidin and detecting the fluorescence using flow cytometry. CXCL12 was used as a positive control to verify that changes in F-actin content in the cells could be detected using this method. CXCL12 (10nM) stimulated an increase in F-actin content in both day 5 and day 12 PBLs at 60 seconds as previously reported (Fig. 4.15; Bleul *et al.*, 1996, Sotsios *et al.*, 1999). This is consistent with its ability to stimulate migration. Neither 2-AG nor JWH-133 stimulated increases in F-actin levels

above vehicle control in day 5 or day 12 PBLs (Figs. 4.16-17). This correlates well with the finding that they do not act as chemoattractants in these cells.

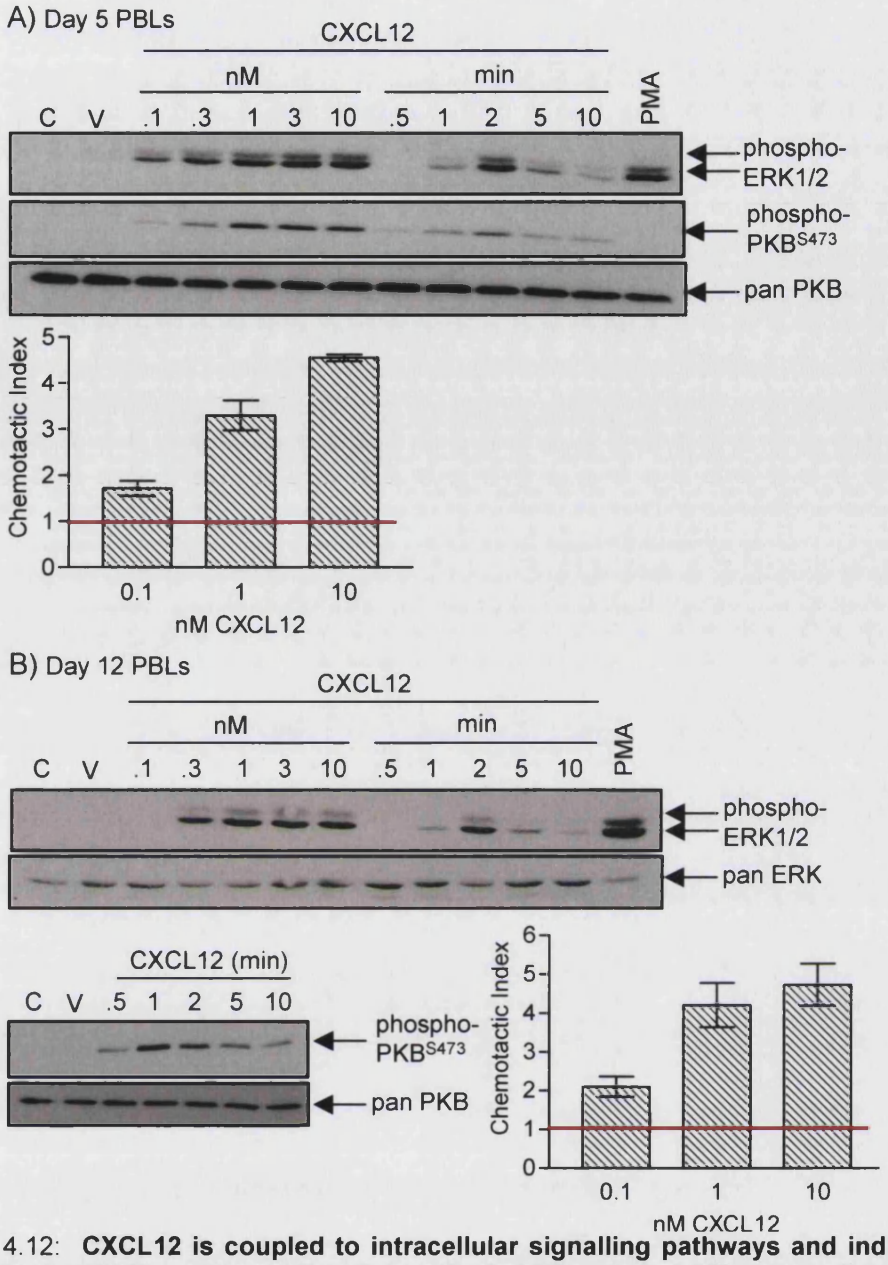


Figure 4.12: **CXCL12 is coupled to intracellular signalling pathways and induces chemotaxis of PBLs.** Day 5 (*A*) and day 12 (*B*) SEB-activated PBLs were stimulated with CXCL12 for either 2 minutes at various concentrations or with 10nM for different lengths of time and cells lysed as described in *Materials and Methods*. Vehicle controls were also carried out (V, 2 minute stimulation with medium). WCLs ($3.2x10^6$ cells per lane) were resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK antibody with affinity for the active Thr202/Tyr204-phosphorylated form of ERK or a phospho-specific PKB antibody with affinity for the Ser473 phosphorylated form of PKB, and visualised with ECL. The blots were stripped and reprobed with pan PKB or pan ERK antibody to verify equal loading and efficiency of protein transfer. Unstimulated cells (control, C) were also lysed to ascertain basal levels of ERK phosphorylation and PMA (100nM, 5 min) was used a positive control. Data are representative of three separate experiments. Cells ($2x10^5$/200µl) were also added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing CXCL12 (0.1-10nM). Chemotaxis across a 5µm pore size membrane was determined after 3 hours as described in *Materials and Methods*. Data are depicted as mean ±S.E.M (n=3). Data was analysed by ANOVA with Bonferroni correction. The red horizontal line at a chemotactic index of 1 represents basal migration and has been included for ease of comparison.

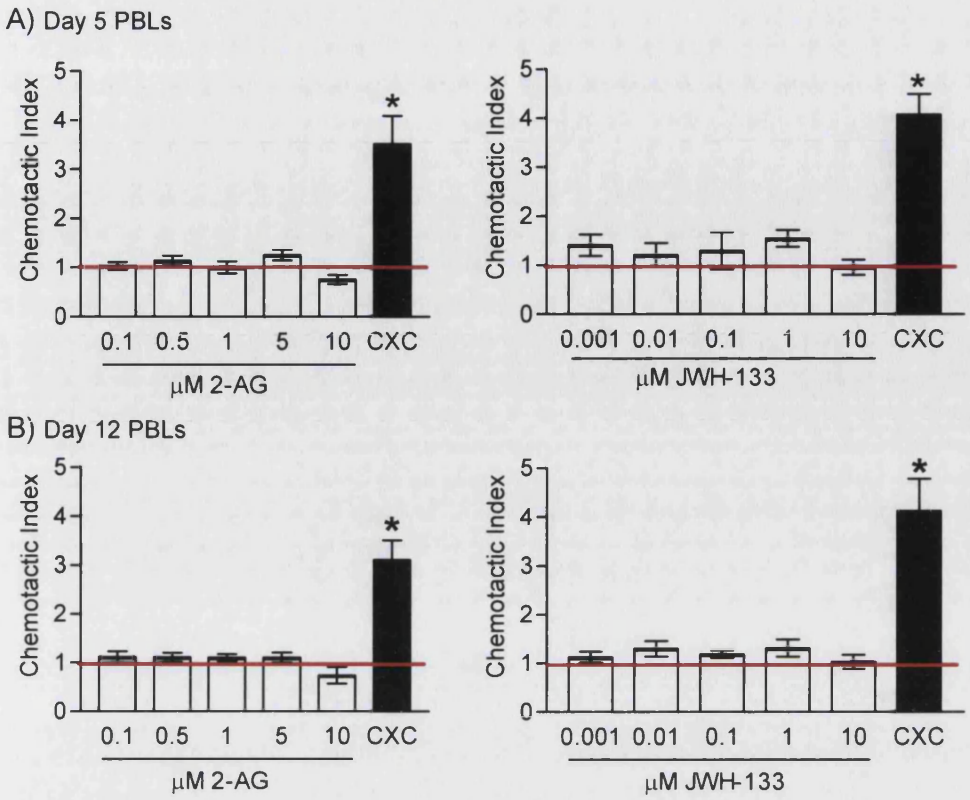


Figure 4.13: **Neither 2-AG nor JWH-133 induces chemotaxis in activated PBLs.** Day 5 (*A*) and day 12 (*B*) SEB-activated PBLs ($2x10^5$/200μl) were added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing 2-AG (0.1-10μM), JWH-133 (0.001-10μM) or CXCL12 (CXC, 10nM). Chemotaxis across a 5μm pore size membrane was determined after 3 hours as described in *Materials and Methods*. Data are depicted as mean ±S.E.M and are derived from three separate experiments (n=3). Data was analysed by ANOVA with Bonferroni correction (*, $p<0.001$ compared to basal migration). The red horizontal line at a chemotactic index of 1 represents basal migration and has been included for ease of comparison.

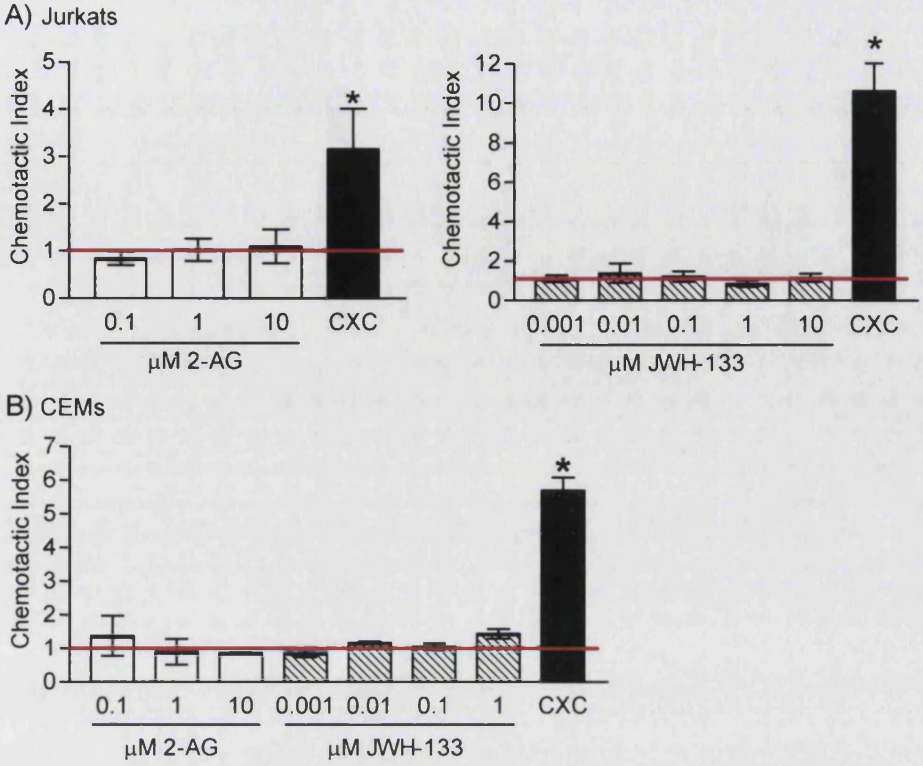


Figure 4.14: **Neither 2-AG nor JWH-133 induces chemotaxis in Jurkats or CEMs.** *A*) Jurkats ($2x10^5$/200μl) were added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing 2-AG (0.1-10μM), JWH-133 (0.001-10μM) or CXCL12 (CXC, 10nM). Chemotaxis across a 5μm pore size membrane was determined after 1.5 hours as described in *Materials and Methods*. Data are depicted as mean ±S.E.M and are derived from three separate experiments (n=3). *B*) CEMs ($2x10^5$/200μl) were added to the upper chamber of a 96-well chemotaxis plate, above lower chambers containing 2-AG (0.1-10μM), JWH-133 (0.001-10μM) or CXCL12 (CXC, 1nM). Chemotaxis across a 5μm pore size membrane was determined after 3 hours as described in *Materials and Methods*. Data are depicted as mean ±S.E.M and are derived from two separate experiments (n=2). Data was analysed by ANOVA with Bonferroni correction (*, $p<0.001$ compared to basal migration). The red horizontal line at a chemotactic index of 1 represents basal migration and has been included for ease of comparison.

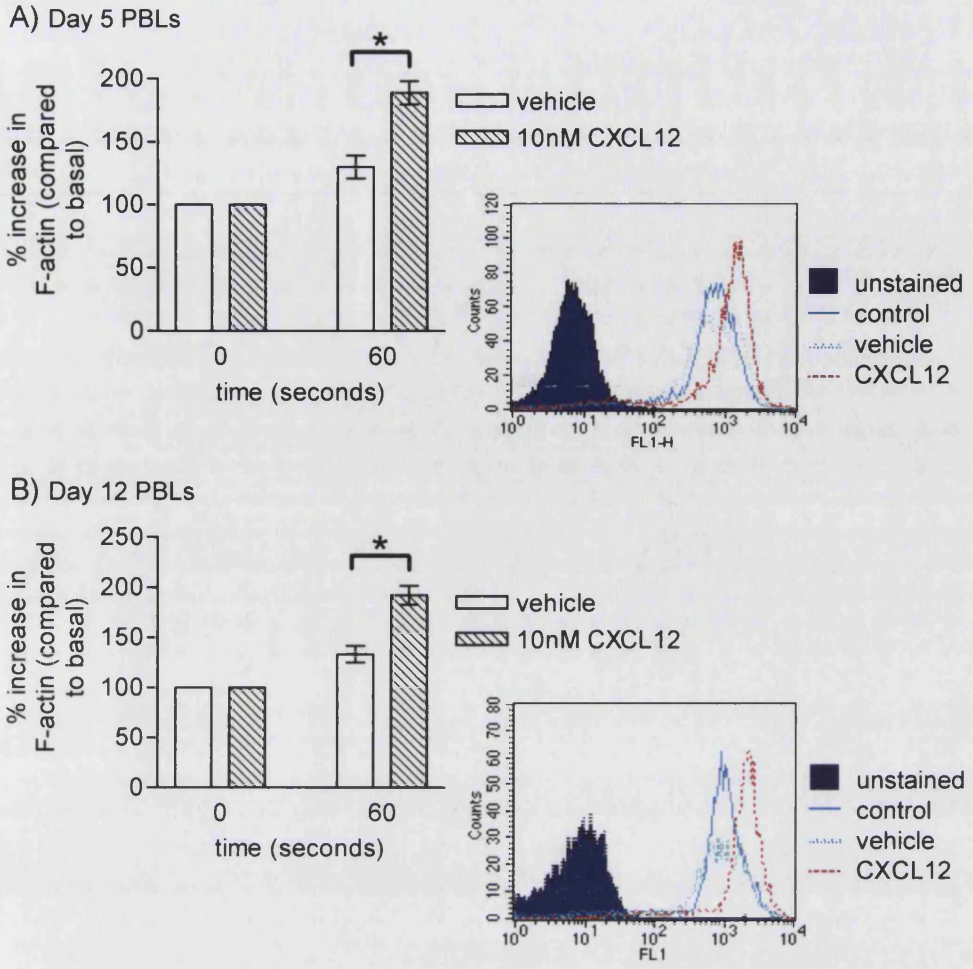


Figure 4.15: **CXCL12 induces robust increases in F-actin content in PBLs.** $2x10^6$ day 5 (*A*) and day SEB-activated 12 (*B*) PBLs were stimulated with 10nM CXCL12 or vehicle (medium) for 60 seconds. The cells were then fixed and permeabilised as described in *Materials and Methods* and stained with FITC-conjugated phalloidin for 30 minutes. Fluorescence was detected using flow cytometry and the median fluorescence intensity of each sample used in analysis. During each experiment samples of unstained and unstimulated (control) cells were also run through the flow cytometer to ascertain autofluorescence and basal levels of F-actin. Data are depicted as mean ±S.E.M and are derived from nine separate experiments pooled together (n=9). Data was analysed by Student's *t* test (*, $p<0.001$). Representative histograms are also shown.

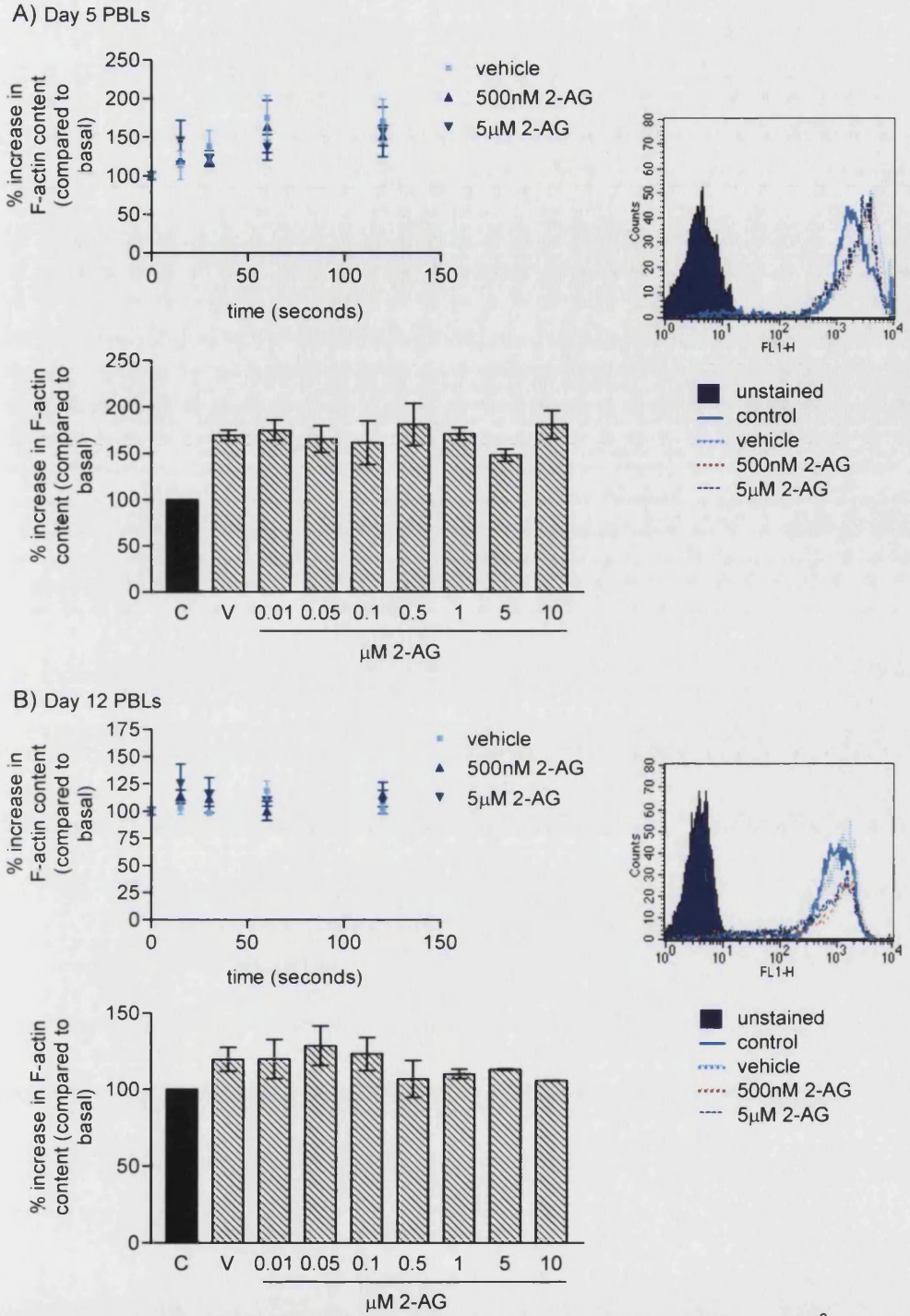


Figure 4.16: **2-AG does not induce actin polymerisation in PBLs.** $2x10^6$ day 5 (*A*) and day 12 (*B*) SEB-activated PBLs were stimulated with varying concentrations (as indicated) of 2-AG for 30 seconds, with 2-AG (500nM or 5μM) for 15-120 seconds or with vehicle (V, ethanol equivalent to 5μM 2-AG) for 30 seconds. The cells were then fixed and permeabilised as described in *Materials and Methods* and stained with FITC-conjugated phalloidin for 30 minutes. Fluorescence was detected using flow cytometry and the median fluorescence intensity of each sample used in analysis. During each experiment samples of unstained and unstimulated (control, C) cells were also run through the flow cytometer to ascertain autofluorescence and basal levels of F-actin. Data are depicted as mean ±S.E.M and are derived from three separate experiments pooled together (n=3). Representative histograms are also shown.

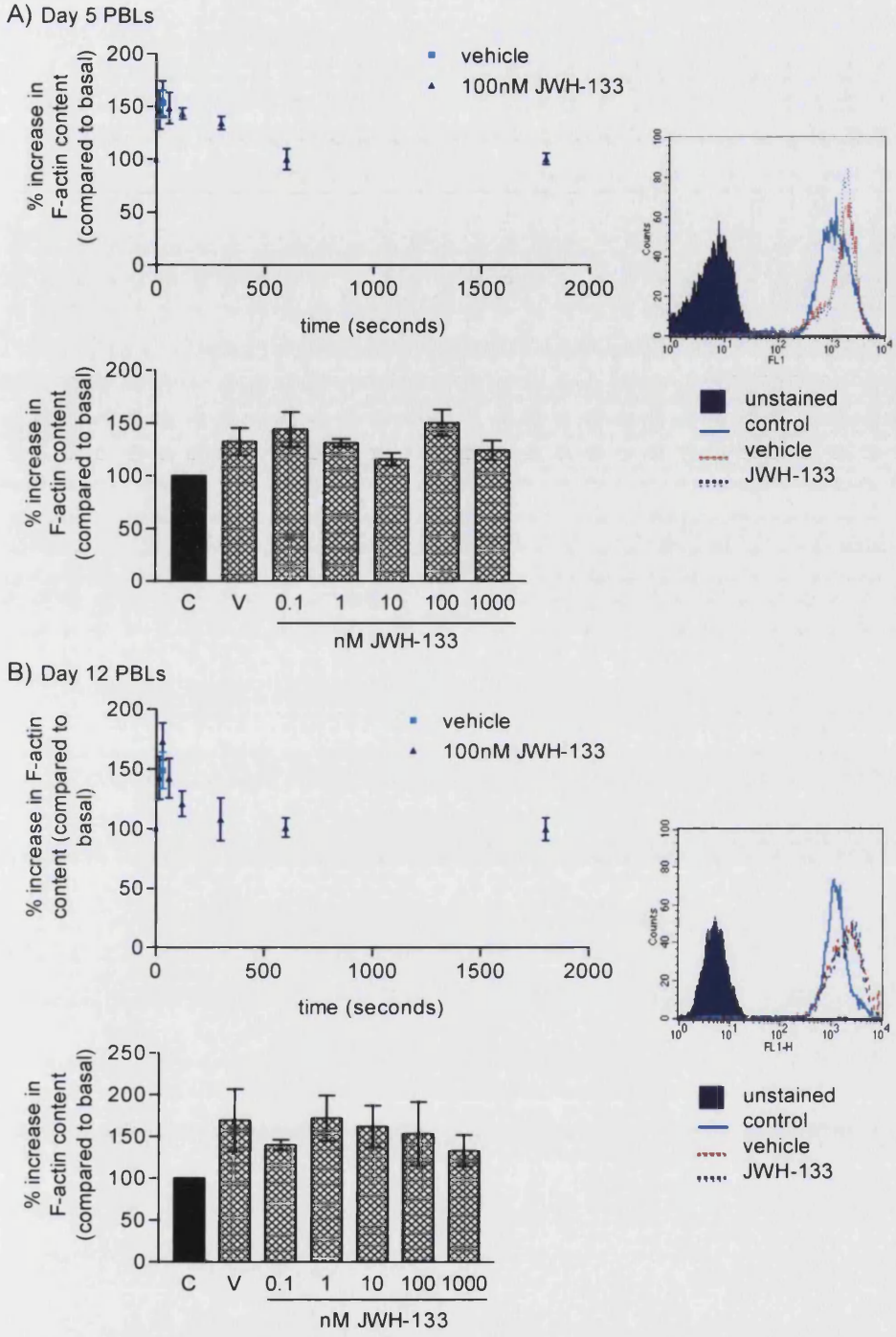


Figure 4.17: **JWH-133 does not induce actin polymerisation in PBLs.** $2x10^6$ day 5 (*A*) and day 12 (*B*) SEB-activated PBLs were stimulated with varying concentrations of JWH-133 for 30 seconds, with WH-133 (100nM) for 15 seconds-30 minutes or with vehicle (V, ethanol equivalent to 100nM JWH-133) for 30 seconds. The cells were then fixed and permeabilised as described in *Materials and Methods* and stained with FITC-conjugated phalloidin for 30 minutes. Fluorescence was detected using flow cytometry and the median fluorescence intensity of each sample used in analysis. During each experiment samples of unstained and unstimulated (control, C) cells were also run through the flow cytometer to ascertain autofluorescence and basal levels of F-actin. Data are depicted as mean ±S.E.M and are derived from three (*A*) or 4 (*B*) separate experiments pooled together (n=3 (*A*), 4 (*B*)). Representative histograms are also shown.

4.3.2 2-AG and JWH-133 inhibit CXCL12-induced chemotaxis of PBLs

In addition to stimulating basal migration, cannabinoids have also been suggested to modulate ligand-induced migration in several leukocytes including rat and murine peritoneal macrophages (Sacerdote *et al.*, 2000, Steffens *et al.*, 2005b) and naïve human $CD8^+$ T lymphocytes (Joseph *et al.*, 2004). To examine whether 2-AG or JWH-133 could modulate ligand-induced migration of PBLs, cells were pre-treated with the cannabinoids and then allowed to migrate towards CXCL12. Both JWH-133 (100nM) and 2-AG (5μM) significantly inhibited CXCL12-induced migration of day 5 and day 12 PBLs (Fig. 4.18). The effect induced by 2-AG is concentration-dependent in day 5 PBLs, with maximal inhibition elicited by 5μM 2-AG (Fig. 4.19).

When CXCL12 is placed in both the upper and lower parts of the chemotaxis chamber, thereby removing the concentration gradient, migration is still increased above basal, indicating that CXCL12 not only induces directed cell migration (chemotaxis) but also increases random cell migration or motility (chemokinesis). Having established that 2-AG inhibits CXCL12-induced migration it was important to assess its relative effects on CXCL12-induced chemotaxis and chemokinesis. When medium alone was placed only in the lower section of the chamber and the cells in the upper chamber simply treated with vehicle, they exhibited some basal migration, with approximately 32,000 cells migrating through the filter (Fig. 4.20). This basal migration was unaffected by any of the pre-treatments carried out on the cells (Fig. 4.20). Addition of CXCL12 to the lower chamber only, induced a robust chemotactic response as predicted (Fig. 4.20). When CXCL12 was placed in both the upper and lower chambers, an increased number of cells migrated to the bottom of the chamber compared to basal, unstimulated levels (Fig. 4.20). However, fewer numbers of cells migrated under such circumstances compared to addition of CXCL12 in the lower chamber alone. Hence, CXCL12 induces both chemokinesis and chemotaxis. When 2-AG was added to the upper chamber, it inhibited the CXCL12-induced chemotactic migration (CXCL12 in lower

chamber only) but had no effect on the CXCL12-induced chemokinetic movement (CXCL12 in upper and lower chambers; Fig. 4.20).

It was next investigated whether 2-AG and JWH-133 were affecting CXCL12-induced chemotaxis at the level of actin polymerisation. Day 5 PBLs were pre-treated with 2-AG, as in the chemotaxis assay, stimulated with CXCL12 and the F-actin content measured as before. Again, CXCL12 elicited a robust increase in F-actin content in the cells and this was indeed inhibited by pre-treating the cells with 2-AG (5μM), consistent with the finding that 2-AG inhibits CXCL12-induced migration. However, the same level of inhibition was also detected when the cells were pre-treated with vehicle (Fig. 4.21). Thus, the inhibition of CXCL12-stimulated actin polymerisation may be an artefact of the experiment and this set of experiments was not pursued further.

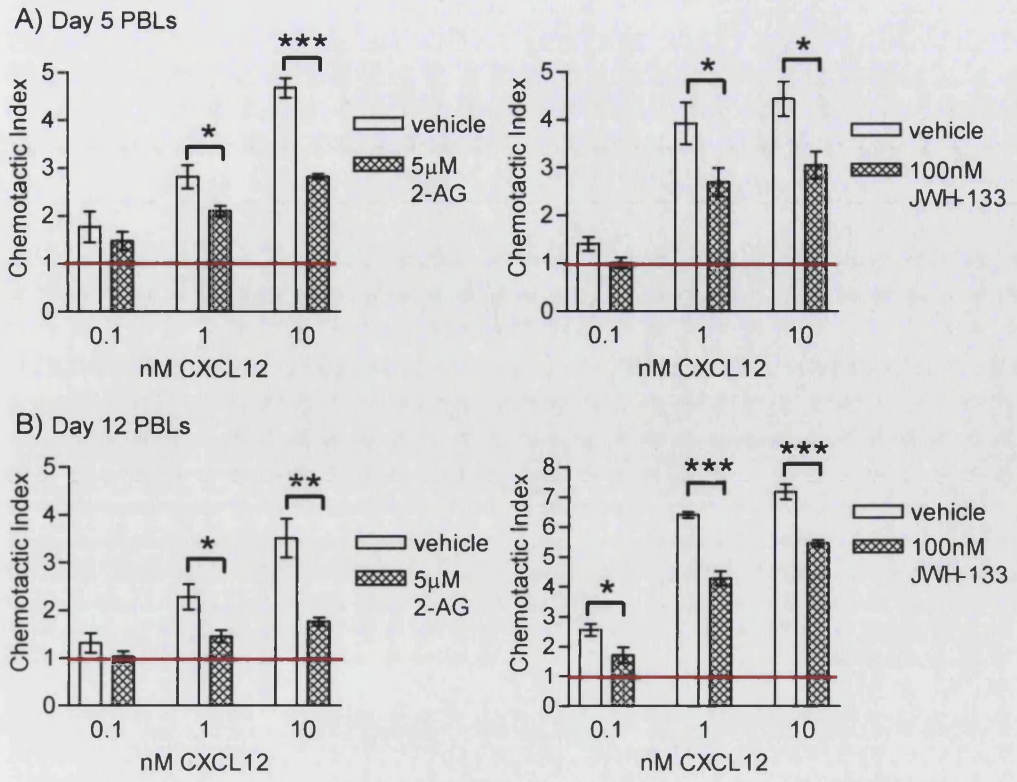


Figure 4.18: **2-AG and JWH-133 inhibit CXCL12-induced chemotaxis in PBLs.** Day 5 (*A*) and day 12 (*B*) SEB-activated PBLs ($2x10^5$/200μl) incubated for 30 minutes at 37°C with either 2-AG (5μM), JWH-133 (100nM) or vehicle (ethanol) were added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing CXCL12 (0.1-10nM). Chemotaxis across a 5μm pore size membrane was determined after 3 hours as described in *Materials and Methods*. Data are depicted as mean ±S.E.M. and are derived from a single experiment (n=4), representative of two others. Data was analysed by Student's *t* test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). The red horizontal line at a chemotactic index of 1 represents basal migration and has been included for ease of comparison.

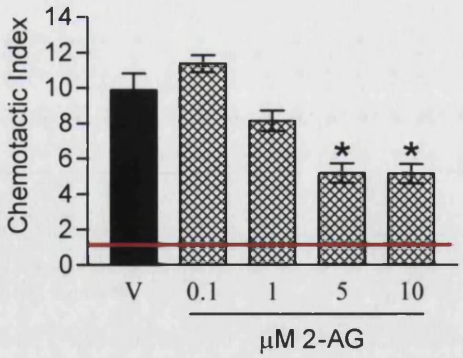


Figure 4.19: **2-AG inhibition of CXCL12-induced chemotaxis in day 5 activated PBLs is concentration-dependent.** Day 5 SEB-activated PBLs ($2x10^5$/200μl) incubated for 30 minutes at 37°C with 2-AG (0.1-10μM) or vehicle (ethanol equivalent to 10μM 2-AG) were added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing 10nM CXCL12. Chemotaxis across a 5μm pore size membrane was determined after 3 hours as described in *Materials and Methods*. Data are depicted as mean ±S.E.M. and are derived from a single experiment (n=6). Data was analysed by ANOVA with Bonferroni correction (*, $p<0.001$ compared to vehicle). The red horizontal line at a chemotactic index of 1 represents basal migration and has been included for ease of comparison.

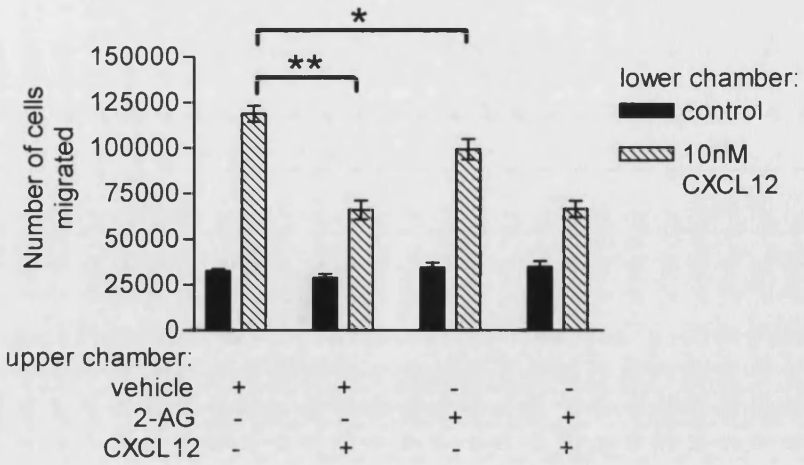


Figure 4.20: **2-AG does not affect CXCL12-induced chemokinesis in day 5 activated PBLs.** Day 5 SEB-activated PBLs were pre-treated with 5µM 2-AG or vehicle (ethanol equivalent to 5µM 2-AG) for 30 minutes at 37°C. After this incubation period CXCL12 (10nM) was added to some of the cell suspension. Cells were then placed in the upper chambers of a 96-well chemotaxis plate above lower chambers containing medium alone (control) or 10nM CXCL12. Chemotaxis across a 5µm pore size membrane was determined after 3 hours as described in *Materials and Methods*. Data are depicted as mean ±SD and are derived from two separate experiments (n=2). Data was analysed by ANOVA with Bonferroni correction (*, $p<0.05$; **, $p<0.001$).

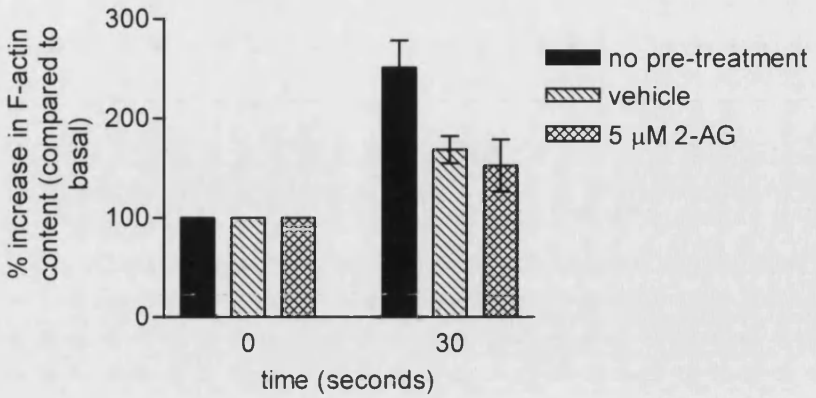


Figure 4.21: **2-AG has no effect on CXCL12-induced increases in F-actin content in day 5 activated PBLs.** Day 5 SEB-activated PBLs were pre-treated with 5μM 2-AG or vehicle (ethanol equivalent to 5μM 2-AG) for 30 minutes. $2x10^6$ cells, with or without pre-treatment, were then stimulated with 10nM CXCL12 for 30 seconds before being fixed and permeabilised as described in *Materials and Methods* and stained with FITC-conjugated phalloidin for 30 minutes. Fluorescence was detected using flow cytometry and the median fluorescence intensity of each sample used in analysis. During each experiment samples of unstained and unstimulated cells were also run through the flow cytometer to ascertain autofluorescence and basal levels of F-actin. Data are depicted as mean ±S.E.M and are derived from three separate experiments (n=3).

4.4 Cannabinoid-induced modulation of T lymphocyte proliferation

4.4.1 JWH-133 effects on IL-2-induced PBL proliferation

It currently remains unclear exactly what effect cannabinoids have on T lymphocyte cell number as reports on the effects of cannabinoids on lymphocyte proliferation have been mixed (Klein *et al.*, 1998a, Croxford & Yamamura, 2005). In order to investigate the effects of cannabinoids on proliferation of activated T lymphocytes, day 5 SEB-activated PBLs were used as this was when CB_2R protein expression was highest. The proliferation assays were carried out in the presence of IL-2 exogenously added IL-2 was first removed for 24 hours prior to the start of the experiment so that all the cells accumulated in the G_0-G_1 phase of the cell cycle.

In the first set of experiments the cells were counted using a Coulter counter after 72 hours of treatment. Samples were set up in duplicate and each sample was run through the machine twice to ensure an accurate reading. The cells are normally maintained in the presence of 50U/ml IL-2 to induce clonal expansion and proliferation. As is clear in Fig. 4.22 at this concentration of IL-2 there was significant proliferation of these cells. However, during the experiment cells were treated with only 25U/ml IL-2 as this concentration of IL-2 still maintains growth of the cells but would hopefully allow any effect of the cannabinoids to be detected as they might be masked at higher concentrations of IL-2. JWH-133 was used in this part of the study as it is a CB_2R-selective agonist and would therefore indicate whether the CB_2R was involved in any responses. JWH-133 stimulated an increase in cell number above that of IL-2 alone (Fig. 4.22*B*) indicating that it is pro-proliferative. The response was not concentration-dependent over the range of concentrations studied.

One study examining the effects of cannabinoids on proliferation of CaCo-2 human colonic epithelial cells re-applied the cannabinoids daily (Ligresti *et al.*, 2003) and therefore it was tested whether applying JWH-133 only once, at the start of the experiment, or daily would have any effect on the response elicited.

The repeated application of JWH-133 made almost no difference (Fig. 4.22*C*). This was not surprising given that JWH-133 should be stable. At 100nM JWH-133 there is a significant difference in cell number between the two types of application, but this is likely to be due to experimental error.

Day 11 SEB-activated PBLs were used to explore whether cells expressing less CB_2R, but the same level of modified CB_1R, as the day 5 PBLs would also respond in the same manner to JWH-133 in terms of proliferation. Day 11, rather than day 12, PBLs were chosen as the cells would not need to be maintained longer than 14 days, which is the normal length of their culture. JWH-133 had no effect on IL-2-induced proliferation of day 11 PBLs (Fig. 4.23*B*). Again, applying JWH-133 only once, compared to daily, had no effect (Fig. 4.23*C*). Notably, these cells on the whole proliferated less, even in response to IL-2, compared to day 5 PBLs (Fig. 4.23*A*).

As day 5 and day 11 PBLs responded differently to JWH-133 the effects of JWH-133, and several other cannabinoids, over a broader concentration range, were explored. Counting the cells using the Coulter counter is a very low throughput assay and therefore XTT assays were used for this. Although the XTT assay does not determine cell number *per se* it still allows a comparison between different experimental conditions to be made and only takes into account viable cells. Once again, in both day 5 and day 11 PBLs IL-2 induced proliferation as an increase in absorbance value was recorded compared to 0U/ml IL-2 (Fig. 4.24*A* and 4.25*A*) reflecting an increase in cell number with increasing concentrations of IL-2. JWH-133 was used again in order to verify whether the responses detected by counting the cells could also be detected by the XTT assay. In addition, ACPA, a CB_1R-selective agonist (Hillard *et al.*, 1999) and met-AEA, a stable AEA analogue (Ramer *et al.*, 2001) were used. Although there was a great deal of variation between experiments, all three cannabinoids appear to induce modest proliferation in day 5 PBLs (Fig. 4.24*B-D*). The cannabinoids also appear to induce an even more modest increase in proliferation in day 11 PBLs (Fig. 4.25*B-D*).

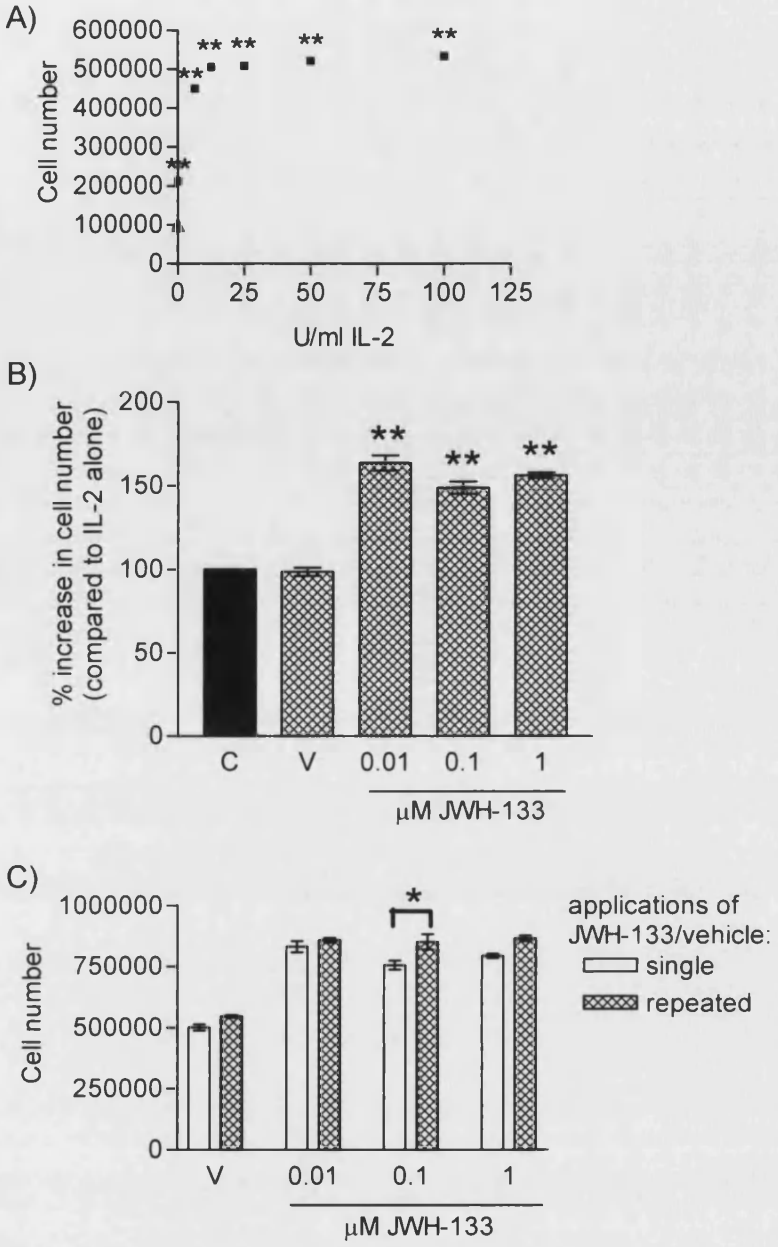


Figure 4.22: **JWH-133 increases IL-2-induced day 5 PBL proliferation as measured using the Coulter counter.** Day 5 SEB-activated PBLs, which had been starved overnight of exogenously added IL-2, were set up at $1x10^5$ cells/well in a 24-well plate and the appropriate concentration of JWH-133 and/or IL-2 added. Each condition was set up in duplicate. After 72 hours the cells were counted using the Coulter Counter as described in *Materials and Methods* with each sample being counted twice. *A*) cells were treated with increasing concentrations of IL-2 (0-100U/ml). ▲represents the number of cells started with. *B,C*) cells were treated with JWH-133 (0.01-1μM) or vehicle (V, ethanol equivalent to 1μM JWH-133) either only once at the start of the experiment (single; *B,C*) or daily (repeated; *C*) in the presence of 25U/ml IL-2. Data are depicted as mean ±S.E.M and are derived from a single experiment (n=4), representative of one other. Data was analysed by ANOVA with Bonferroni correction (*, $p<0.05$; **, $p<0.001$ compared to the number of cells started with (*A*) or control (*B*)).

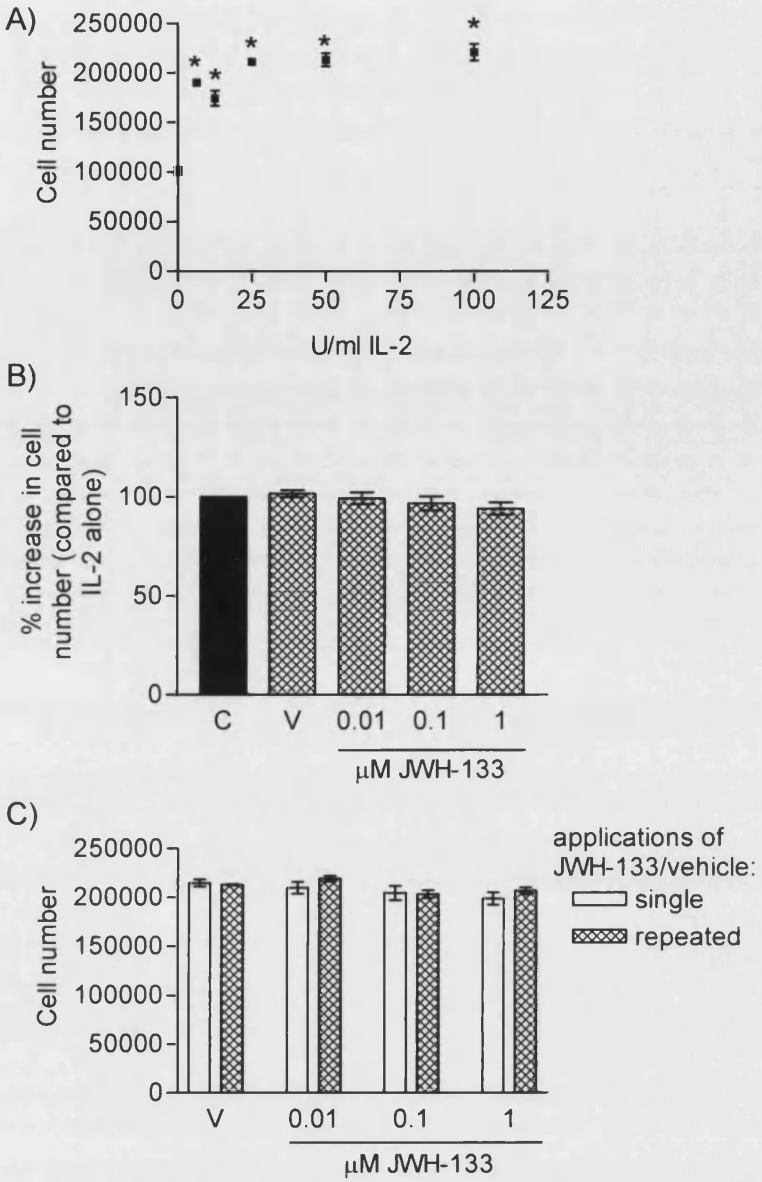


Figure 4.23: **JWH-133 has no effect on IL-2-induced day 11 PBL proliferation as measured using the Coulter counter.** Day 11 SEB-activated PBLs, which had been starved overnight of exogenously added IL-2, were set up at $1x10^5$ cells/well in a 24-well plate and the appropriate concentration of JWH-133 and/or IL-2 added. Each condition was set up in duplicate. After 72 hours the cells were counted using the Coulter Counter as described in *Materials and Methods* with each sample being counted twice. *A*) cells were treated with increasing concentrations of IL-2 (0-100U/ml). *B,C*) cells were treated with JWH-133 (0.01-1µM) or vehicle (V, ethanol equivalent to 1µM JWH-133) either only once at the start of the experiment (single; *B,C*) or daily (repeated; *C*) in the presence of 25U/ml IL-2. Data are depicted as mean ±S.E.M and are derived from a single experiment (n=4), representative of one other. Data was analysed by ANOVA with Bonferroni correction (*, $p<0.001$ compared to the number of cells started with).

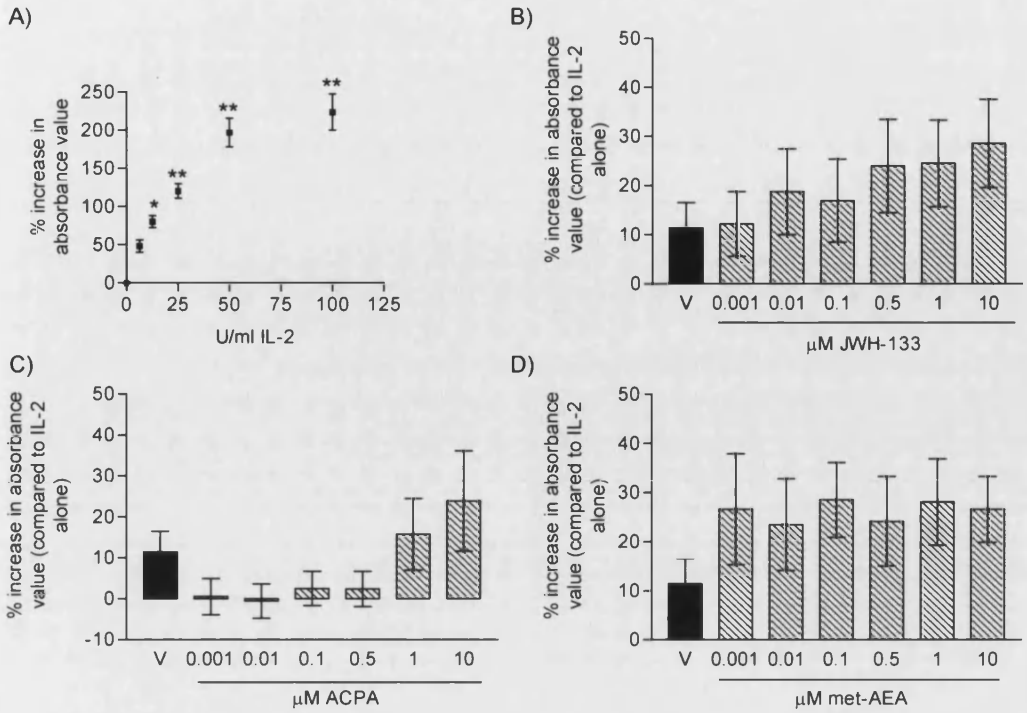


Figure 4.24: **Cannabinoids modestly increase IL-2-induced day 5 PBL proliferation as measured by XTT assay.** Day 5 SEB-activated PBLs, which had been starved overnight of exogenously added IL-2, were set up at a density of $5x10^4$ cells/well in a 96-well tray and the appropriate concentration of cannabinoid and/or IL-2 added. After 72 hours, PMT-XTT solution was added to the wells and the plate allowed to develop. Changes in absorbance were measured using a plate reader. *A*) cells were treated with increasing concentrations of IL-2 (0-100U/ml). Cells were treated with 0.001μM-10μM JWH-133 (*B*), ACPA (*C*), met-AEA (*D*) or vehicle (V, ethanol) for 72 hours in the presence of 25U/ml IL-2. Data are depicted as mean ±S.E.M and are derived from 4 separate experiments pooled together (n=12). Data was analysed by ANOVA with Bonferroni correction (*, $p<0.01$; **, $p<0.001$ compared to 0U/ml IL-2).

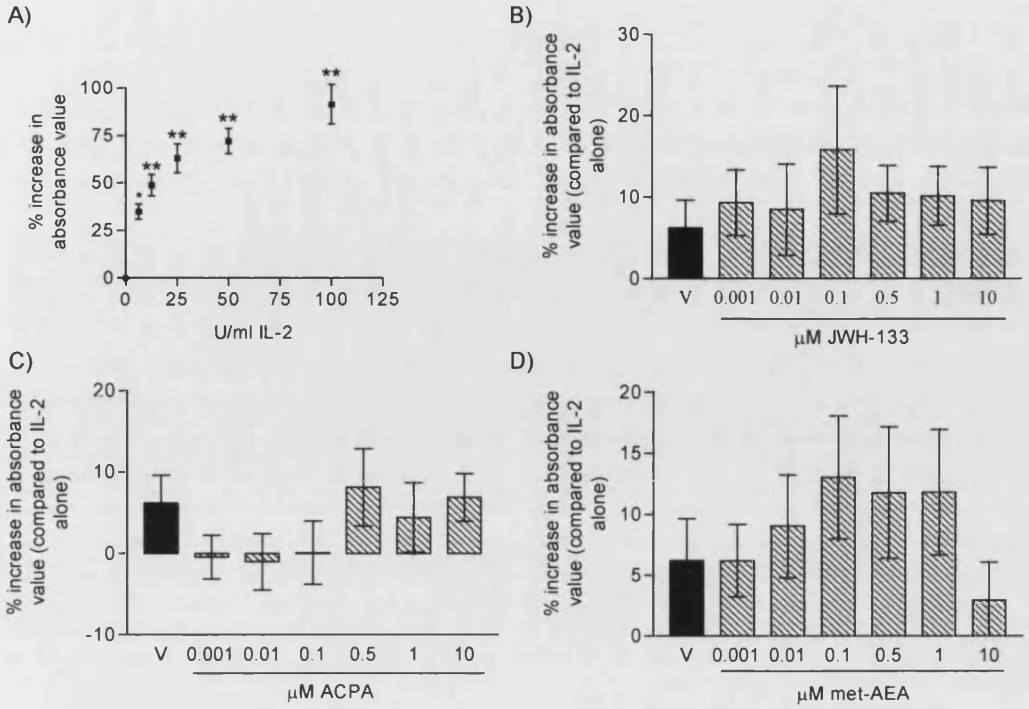


Figure 4.25: **Cannabinoids have little effect on IL-2-induced day 11 PBL proliferation as measured by XTT assay.** Day 11 SEB-activated PBLs, which had been starved overnight of exogenously added IL-2, were set up at a density of $5x10^4$ cells/well in a 96-well tray and the appropriate concentration of cannabinoid and/or IL-2 added. After 72 hours, PMT-XTT solution was added to the wells and the plate allowed to develop. Changes in absorbance were measured using a plate reader. *A*) cells were treated with increasing concentrations of IL-2 (0-100U/ml). Cells were treated with 0.001μM-10μM JWH-133 (*B*), ACPA (*C*), met-AEA (*D*) or vehicle (V, ethanol) for 72 hours in the presence of 25U/ml IL-2. Data are depicted as mean ±S.E.M and are derived from 4 separate experiments pooled together (n=12). Data was analysed by ANOVA with Bonferroni correction (*, $p<0.05$; **, $p<0.001$ compared to 0U/ml IL-2).

4.5 Summary

- AEA, 2-AG and JWH-133 induce ERK1/2 phosphorylation in day 5 and 12 SEB-activated PBLs and Jurkats. This response was determined to be receptor mediated as a Gαi/o-protein was involved, but the roles of the modified CB_1R and CB_2R specifically could not be determined.
- In the day 5 PBLs it was determined that AEA-stimulated ERK1/2 phosphorylation is MEK and PKC-dependent.
- 2-AG was also shown, in day 5 PBLs, to elevate levels of forskolin-induced cAMP production.
- Neither 2-AG nor JWH-133 induce migration of day 5 or 12 SEB-activated PLBs but inhibit CXCL12-induced chemotaxis of these cells.
- JWH-133 exerts a pro-proliferative effect on day 5 but not day 11 SEB-activated PBLs.

4.6 Discussion

The main aim of this part of the study was to investigate the effects that cannabinoids have on the function of activated T lymphocytes. However, first it was important to establish whether the receptors detected in activated PBLs were biochemically functional by examing cannabinoid-induced activation of a signalling pathway, namely ERK1/2.

4.6.1 AEA, 2-AG and JWH-133 induce ERK1/2 phosphorylation

Cannabinoids have been shown to positively couple to a number of downstream intracellular signalling pathways, including the phosphorylation and activation of ERK1/2 in a number of different cell types (Bouaboula *et al.*, 1995b, Bouaboula *et al.*, 1996, Galve-Roperh *et al.*, 2002, Derkinderen *et al.*, 2003, Samson *et al.*, 2003). However, there have also been reports of cannabinol, one of the natural cannabinoids, not stimulating acute ERK phosphorylation and inhibiting PMA/Ionomycin-induced activation of nuclear ERK phosphorylation in murine splenocytes (Faubert & Kaminski, 2000, Faubert Kaplan & Kaminski, 2003).

In this study both the endocannabinoids AEA and 2-AG and the CB_2R-selective agonist JWH-133 induced acute ERK1/2 phosphorylation in day 5 and 12 SEB-activated PBLs and Jurkats. Given that cannabinoids have been reported to positively couple to ERK activation, including in Jurkats (Herrera *et al.*, 2005, Ghosh *et al.*, 2006), the ability of AEA, 2-AG and JWH-133 to stimulate ERK1/2 phosphorylation in PBLs was not unexpected.

4.6.1.1 Is cannabinoid-induced ERK1/2 phosphorylation in activated PBLs and Jurkats receptor-mediated?

In *Results I* it was shown that by day 12 post-isolation the PBLs still express a modified CB_1R but very little CB_2R protein. Thus, the fact that the cannabinoids continue to couple to ERK1/2 phosphorylation in these cells is an indication that either: i) the modified CB_1R is functional and coupled to biochemical pathways, ii) that very low CB_2R occupancy is required to generate a signal, iii) that the cannabinoids are acting via non $CB_{1/2}R$ cannabinoid-like receptors or iv) that the cannabinoids are acting via cannabinoid receptor-independent mechanisms.

The data presented in this study indicate that the modified CB_1R may not be functional. Although AEA and 2-AG stimulated ERK1/2 phosphorylation in both day 5 and day 12 PBLs neither endocannabinoid induced this response in naïve T lymphocytes. In the first part of this study only a modified CB_1R protein, but not the CB_2R, was detected in these cells, suggesting that this modified receptor is either not functional or is but does not couple to ERK1/2 activation. The ability of AEA and 2-AG to phosphorylate another kinase, PKB, which has been shown to lie downstream of cannabinoids (Gomez *et al.*, 2000, Galve-Roperh *et al.*, 2002, Molina-Holgado *et al.*, 2002, Sanchez *et al.*, 2003) was also examined. Again, neither endocannabinoid stimulated an increase in PKB^{Ser473} phosphorylation, suggesting that the modified CB_1R may not be functional although additional signalling pathways need to be studied in order to verify this.

As the modified CB_1R does not appear to be coupled to ERK1/2 phosphorylation the question remains whether the cannabinoids are acting through the CB_2R in activated PBLs and Jurkats. Other studies have reported that low receptor occupancy is required for cannabinoid effects. For instance, 95% maximal inhibition of acetylcholine release by the synthetic cannabinoid WIN55212-2 in rat hippocampal slices occurs at only 7.5% CB_1R occupancy (Gifford *et al.*, 1999) and 2-AG and noladin ether were shown to require occupancy of less than 50% of the CB_2R expressed on transfected CHO cells to regulate ERK activation (Shoemaker *et al.*, 2005b). Thus, it appears that cannabinoids could couple to signalling pathways even if low levels of receptor proteins are expressed which could explain why the cannabinoids continue to induce ERK1/2 phosphorylation even in day 12 PBLs when CB_2R expression is low.

The fact that AEA and 2-AG-induced ERK1/2 phosphorylation is mimicked by JWH-133 also supports the notion that they are acting via the CB_2R as JWH-133 is a CB_2R-specific agonist with a Ki of 3.4nM at the CB_2R compared to 677nM at the CB_1R (Howlett *et al.*, 2002). However, it must be highlighted that it is CB_2R-selective rather than specific and at the higher concentrations used here, such as 100nM, may also be activating the CB_1R and possibly additional receptors.

Thus, several inhibitors were used in order to investigate whether the cannabinoids were acting in a CB_2R-dependent manner. Pre-treatment of the cells with the Gαi/o-protein inhibitor PTX abolished cannabinoid-induced ERK1/2 phosphorylation in day 5 and 12 PBLs indicating that a Gi/o-protein was involved. This is consistent with a possible role for the CB_2R but does not rule out involvment of other receptors such as the abn-cannabidiol sensitive receptor (Begg *et al.*, 2005). In fact, a putative role for such an additional receptor is supported by the finding that AEA, which is generally considered to be a weak CB_2R agonist, and can even act as an antagonist (Gonsiorek *et al.*, 2000), is coupled to ERK1/2 phosphorylation in these cells. Furthermore, in Jurkats, PTX did not completely abrogate the response to AEA, 2-AG or JWH-133, suggesting that there may also be a role for a PTX-insensitive receptor. The recent finding that the orphan GPCR GPR55, which does not couple to Gi-proteins, may be an

additional cannabinoid receptor could account for this. However, this is unlikely as it has been shown that JWH-133 does not stimulate GPR55 (Baker *et al.*, 2006).

Two antagonists, AM251, a CB_1R specific antagonist and AM630, a CB_2R specific antagonist (Palmer *et al.*, 2002) were also used to examine the role of the cannabinoid receptors in the ERK1/2 responses. Unfortunately, the effects detected with these two compounds were mimicked by the vehicle controls and the results were thus inconclusive. It is not clear why the vehicle controls mimicked the effects of these inhibitors as in each case the levels of vehicle, either ethanol or DMSO, were very low. Other antagonists do exist, such as the SR141716A and SR144528 compounds, and these could be used to further this work. In addition, the development of new water-soluble cannabinoids has been reported (Pertwee *et al.*, 2000, Martin *et al.*, 2006). Such antagonists should avoid any problems with vehicle control. Alternatively, siRNA could be used to silence the individual receptors to elucidate what role they play and also whether the modified CB_1R is functional.

Thus, at present it is unclear through which receptor the cannabinoids are coupling to ERK1/2 phosphorylation in activated T lymphocytes and further work is required to determine whether the CB_2R and/or modified CB_1R play a role.

4.6.1.2 Are the effects of AEA and 2-AG mediated by their metabolites?

Physiologically both AEA and 2-AG are rapidly taken up by cells and are degraded by a number of enzymes including FAAH and MAGL (Goparaju *et al.*, 1998, Cravatt & Lichtman, 2002, Dinh *et al.*, 2002). Both of these enzymes were found to be expressed in PBLs and Jurkats in *Results I.* Hence, it was investigated whether AEA and 2-AG degradation affects their ability to stimulate ERK1/2 phosphorylation, even over the short time course studied here. The potent FAAH and MAGL inhibitor MAFP was used (De Petrocellis *et al.*, 1997,

Goparaju *et al.*, 1999, Saario *et al.*, 2004). Initial experiments revealed that when day 12 PBLs or Jurkats were pre-treated with MAFP, AEA and 2-AG-induced ERK1/2 phosphorylation was attenuated. Given that MAFP should be preventing AEA and 2-AG degradation when these cannabinoids come into contact with the cells, this response suggests that what was assumed to be AEA and 2-AG-induced ERK1/2 phosphorylation is actually caused by AEA and 2-AG metabolites. However, MAFP has also been shown to bind irreversibly to the CB_1R (Deutsch *et al.*, 1997) and act as a CB_1R antagonist (Fernando & Pertwee, 1997). To my knowledge there have been no reports published concerning its effects on the CB_2R. Therefore, MAFP could be preventing the binding of AEA and 2-AG to the modified CB_1R and hence attenuating the ERK1/2 response. The latter theory does require the modified CB_1R to be functional, which appears not to be the case.

Further investigation was carried out in day 5 PBLs and showed the same downregulation of AEA-induced ERK1/2 phosphorylation with MAFP pre-treatment as in the day 12 PBLs and Jurkats. However, the response was not concentration-dependent and more importantly was also mimicked by vehicle control. Furthermore, MAFP also abolished JWH-133-induced ERK1/2 phosphorylation.

This additional data suggests that the response to MAFP detected was an artefact and that AEA and 2-AG stimulation of ERK1/2 phosphorylation in T lymphocytes is likely to be mediated by the cannabinoids themselves rather than their metabolites. However, it must be remembered that AEA and 2-AG are not simply degraded by FAAH and MAGL but that other metabolic pathways exist and these should also investigated before firm conclusions are drawn. For instance, both AEA and 2-AG are oxygenated by COX-2 to produce a range of prostamides and prostaglandin-glycerol esters, respectively (Kozak *et al.*, 2002a) and it has been reported that such metabolites may exert their own effects. For example, PGE_2-G was reported to trigger Ca^{2+} mobilisation and increase levels of ERK1/2 phosphorylation in a PKC-dependent manner in the murine macrophage-like cell line, RAW264.7 (Nirodi *et al.*, 2004). Similarly, AEA and 2-AG can also be metabolised by lipoxygenases and 15-HETE-G has been

shown to activate PPAR-α (Kozak *et al.*, 2002b). As COX-2 has been reported to be expressed at low levels in naïve T lymphocytes and at higher levels upon activation (Pablos *et al.*, 1999) and 5-lipoxygenase also appears to be expressed in primary T lymphocytes (Los *et al.*, 1995), the effect of COX-2 and lipoxygenase inhibitors on AEA and 2-AG-induced phosphorylation of ERK1/2 in T lymphocytes should be also be examined.

4.6.1.3 Why are such high concentrations of AEA and 2-AG required to induce ERK1/2 phosphorylation?

Maximal stimulation of ERK1/2 phosphorylation with either AEA or 2-AG occurred at 10μM although their reported Ki values are much lower than this for both receptors. This has been seen in other studies and it has often brought into question whether these responses would occur physiologically given that reported concentrations of AEA and 2-AG in most tissues are in the pmol/g and nmol/g range, respectively (Goutopoulos & Makriyannis, 2002, Sugiura *et al.*, 2002). One argument is that local concentrations of AEA and 2-AG may in fact be higher than thought. Both endocannabinoids act as autocrine or paracrine mediators and they are also hydrophobic, so their concentration at the lipid bilayer, near the receptors, may be higher than in the extracellular environment (Ambrosi *et al.*, 2005). Furthermore it is know that levels can rise during, for instance, pathological conditions (Goutopoulos & Makriyannis, 2002, Sugiura *et al.*, 2002). Regulatory mechanisms may also exist *in vivo* that may affect the concentrations of cannabinoid required to activate the receptors. For instance, the concentration of AEA required to activate the VR1 is reduced by factors such as phosphorylation by PKC (Ross, 2003).

It is also possible that the high concentrations required in the experiments presented here and in other studies to stimulate maximal responses are due to experimental conditions. For example, 2-AG is known to be relatively unstable in media and is quickly converted to 1(3)-AG (Rouzer *et al.*, 2002). Thus, even though every care was taken to prepare and use 2-AG as quickly as possible in every experiment, it is possible that what was believed to represent 10μM was

actually less. Furthermore, both 2-AG and AEA have been reported to adhere to plastics (Rouzer *et al.*, 2002, Karlsson *et al.*, 2004, Ortega-Gutierrez *et al.*, 2004). Therefore, it is possible that the actual concentrations of AEA and 2-AG that reaches the cells are lower than thought.

4.6.1.4 Several kinases couple the cannabinoids to ERK1/2 activation in activated PBLs and Jurkats

A number of different pathways can link GPCRs to ERK activation (Marinissen & Gutkind, 2001, Werry *et al.*, 2005), therefore several inhibitors were used to determine some of the kinases upstream of ERK1/2 phosphorylation by AEA in day 5 SEB-activated PBLs. MEK is the dual specificity kinase directly upstream of ERK (Kolch, 2000) and its involvement was verified using the MEK1 inhibitor PD98059 (Alessi *et al.*, 1995). AEA-induced ERK1/2 phosphorylation was shown to be MEK dependent in day 5 PBLs and Jurkats.

Cannabinoid-induced ERK activation was also shown to be PKC-dependent in CB_2R-transfected CHO cells and ECV304 human umbilical vein endothelial cells (Bouaboula *et al.*, 1996, Liu *et al.*, 2000). The PKC inhibitor Ro-32-0432, at concentrations were it would not be selective for any one isoform (Wilkinson *et al.*, 1993), suppressed the AEA-induced response in day 5 PBLs indicating that PKC lies upstream of ERK1/2 phosphorylation. Two additional inhibitors were used to further investigate whether AEA activated particular PKC isoforms. T lymphocytes express PKCα, β, δ, ε, θ, and ζ isoforms (Tan & Parker, 2003). Rottlerin, at 10μM, a concentration which should selectively inhibit PKCδ compared to other PKC isorms (Gschwendt *et al.*, 1994, Villalba *et al.*, 1999), attenuated AEA-induced ERK1/2 phosphorylation, indicating that AEA exerts at least some of its actions through this PKC isoform. It is important to note that at 10μM rottlerin can also inhibit another kinase, calcium/calmodulin-dependent protein kinase (CaM kinase) III. Cannabinol has been shown to couple to another member of the CaM kinase family, CaM kinase II (Jan & Kaminski, 2001), which has also been linked to ERK activation in other systems (English *et al.*, 1999). Thus it is not possible at this time to rule out a role for CaM kinase III

in the AEA-induced ERK1/2 response. AEA did not appear to activate PKCα or βI as treatment of the cells with the inhibitor Gö6976, which is selective for these isoforms over the concentrations used (Martiny-Baron *et al.*, 1993), had no effect on ERK1/2 phosphorylation. The relevance of AEA activating PKC, apart from its involvement in ERK1/2 phosphorylation and activation, is that PKC phosphorylation of the CB_1R blocks ion channel modulation, thus activation of PKC downstream of the cannabinoids could be part of a negative feedback loop to control receptor activation (Garcia *et al.*, 1998). This may not be critical in T lymphocytes which only express a modified CB_1R, which may in fact not be active, but may be more relevant in other cell types.

Another kinase shown to be upstream of ERK in cannabinoid-induced signalling is PI3K (Bouaboula *et al.*, 1997, Galve-Roperh *et al.*, 2002, Samson *et al.*, 2003, Sanchez *et al.*, 2003). The involvement of PI3K in AEA and 2-AG-induced ERK1/2 phosphorylation in Jurkats was investigated using the non-selective PI3K inhibitor LY294002 (Ward *et al.*, 2003). The results indicate a role for PI3K in AEA-induced ERK phosphorylation and to a lesser extent 2-AG-induced ERK1/2 phosphorylation. This suggests that the role of PI3K may be partly agonist dependent. Such agonist-directed trafficking of responses by cannabinoids has previously been shown in CHO-CB_2R cells. Shoemaker *et al* (2005b) showed that whilst 2-AG, noladin ether and CP55,940 each regulated three different intracellular pathways, namely AC inhibition, ERK stimulation and induction of Ca^{2+} transients, with equivalent efficacy, the rank order of potencies differed depending on the pathway studied.

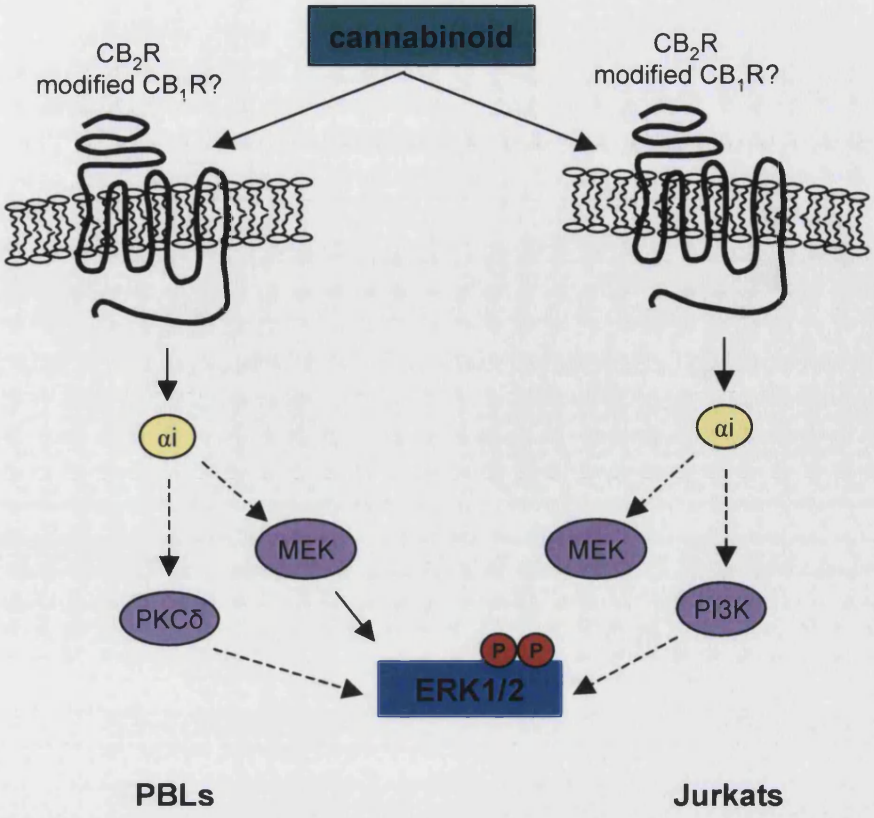


Figure 4.26: **Model of ERK1/2 activation in PBLs and Jurkats.** Currently it is not clear whether the modified CB_1R detected in these cells in functional or not but cannabinoid-induced ERK1/2 phosphorylation in these cells is likely to be CB_2R-mediated. In day 5 SEB-activated PBLs it was shown that AEA-induced ERK1/2 phosphorylation is MEK and PKC-dependent whilst in Jurkats a role for PI3K was elucidated. These results corroborate data from other studies.

4.6.2 2-AG appears to enhance forskolin-induced cAMP production in activated PBLs

Another pathway that was briefly studied was cannabinoid modulation of acute cAMP production. This was one of the first pathways downstream of the cannabinoids to be defined and it has been demonstrated that although cannabinoids generally inhibit forskolin-induced cAMP production (Pertwee, 1997, Kaminski, 1998, Howlett *et al.*, 2002, Demuth & Molleman, 2006), they can also increase forskolin-induced (Glass & Felder, 1997, Felder *et al.*, 1998) and basal (Maneuf & Brotchie, 1997, Steffens *et al.*, 2005a) cAMP production. These differences are suggested to result from differential coupling of the CB_1R to Gi/o and Gs-proteins (Glass & Felder, 1997, Bonhaus *et al.*, 1998, Jarrahian *et al.*, 2004) and the specific isoform distribution of AC (Rhee *et al.*, 1998). Δ^9-

THC has been shown to suppress forskolin-stimulated cAMP production in human PBMCs as well as the murine thymoma-derived T lymphocyte cell line EL4.IL-2 (Diaz *et al.*, 1993, Condie *et al.*, 1996) and 2-AG inhibited forskolin-induced cAMP production in murine splenocytes (Mechoulam *et al.*, 1995). Further evidence that cannabinoids can inhibit cAMP production in T lymphocytes has come from studies examining the effects of cannabinoids on CRE, a part of the cAMP signalling cascade downstream of PKA. Several cannabinoids, including Δ^9-THC and 2-methylarachidonyl-(2'fluoroethyl)amide, a stable AEA analogue, inhibit CRE DNA binding activity in murine thymocytes and splenocytes (Koh *et al.*, 1997, Herring & Kaminski, 1999, Kaplan *et al.*, 2005b).

In this study, preliminary evidence suggests that neither JWH-133 nor 2-AG affect basal cAMP levels. However, 2-AG robustly increased forskolin-induced cAMP production. This effect was not mimicked by the CB_2R-selective agonist JWH-133, suggesting that this response is not CB_2R-mediated and that 2-AG is acting through an alternative mechanism. Stimulation of cAMP production is associated with Gs-protein activity and the CB_2R does not couple to this G-protein (Glass & Felder, 1997), corroborating the idea that 2-AG is not acting through the CB_2R. 2-AG may instead be acting via the modified CB_1R expressed in these cells. There have been reports that the CB_1R can couple to Gs-proteins (Glass & Felder, 1997, Bonhaus *et al.*, 1998, Jarrahian *et al.*, 2004), which would account for the increase in forskolin-induced cAMP stimulated by 2-AG. However, this is purely speculative at this stage as it remains to be determined whether the modified CB_1R is functional. Cannabinoids have been shown to exert several receptor-independent effects, including the stimulation of cAMP production. For example, it has previously been reported when AEA and Δ^9-THC increased forskolin-induced cAMP levels in both CHO-K1 wild type and CB_2R-transfected cell (Slipetz *et al.*, 1995). Thus 2-AG could be acting in a receptor-independent manner in this assay. Regardless of the mechanism, 2-AG is likely to be coupling to AC-VII to induce the increase in cAMP production detected. Both AC-VI and AC-VII are ubiquitously expressed (Sunahara & Taussig, 2002) and activation of the cannabinoid receptors has been shown to inhibit AC-VI but stimulate AC-VII (Rhee *et al.*, 1998). This suggests that 2-AG

is probably coupling to AC-VII in the day 5 PBLs. These experiments would need to be repeated and also extended to verify how 2-AG may be exerting its effect on cAMP production.

4.6.3 What are the effects of JWH-133 and 2-AG on migration of activated PBLs?

Having established that cannabinoids couple to intracellular signalling pathways in activated PBLs the next stage of this study was to determine their effects on T lymphocyte functions. Several reports have suggested that cannabinoids affect spontaneous cell migration. For instance, it has been reported that 2-AG acts as a chemoattractant for immune cells including HL-60 cells differentiated into macrophage-like cells (Kishimoto *et al.*, 2003), CB_2R-expressing myeloid precursor cells (Jorda *et al.*, 2003), B lymphocytes (Jorda *et al.*, 2002, Rayman *et al.*, 2004) and eosinophils (Oka *et al.*, 2004) and the CB_2R was implicated in these responses. Although 2-AG is generally considered to induce migration, results using other cannabinoids have been mixed, indicating that cannabinoid effects on basal migration may be both ligand and cell-type specific.

4.6.3.1 Neither 2-AG nor JWH-133 induce migration of activated PBLs

In this study, 2-AG did not induced chemotaxis in day 5 or 12 PBLs, although these were all shown to be capable of migrating towards the chemokine CXCL12. As this contradicts several reports which suggest that 2-AG is a chemoattractant, this data was verified in two T lymphocyte cell lines, Jurkats and CEMs. Once again 2-AG did not induce chemotaxis in these cells although the cells migrated towards CXCL12. Thus, 2-AG does not act as a chemoattractant for T lymphocytes.

Although in general 2-AG has been shown to act as a chemoattractant there has been a report that it has no effect on spontaneous migration of human undifferentiated HL-60 cells (Kishimoto *et al.*, 2003). Both HL-60 cells and the

T lymphocytes used in the current study express the CB_2R, which has been implicated in the chemoattractant properties of 2-AG. Therefore, it may be that the effects of 2-AG are cell-type specific. Notably, it has been established that 2-AG is rapidly converted to 1(3)-AG in media (Rouzer *et al.*, 2002) and it could be argued that the lack of response to 2-AG is due to its conversion to 1(3)-AG over the 3 hour assay. However, this same conversion would also have occurred in all the studies which conclude that 2-AG is a chemoattractant as a similar, or longer, time frame was used in each instance. In the future it will be important to determine whether the effects of 2-AG on cell migration detected in these *in vitro* studies are due to 2-AG itself or 1(3)-AG, especially as 1(3)-AG has been shown to exert its own effects (Stella *et al.*, 1997).

JWH-133, a CB_2R-selective agonist, also did not induce migration of PBLs, Jurkats or CEMs, which extends and confirms the report published recently which suggests that AEA and JWH-133 do not induce migration of naïve human $CD8^+$ T lymphocytes (Joseph *et al.*, 2004). JWH-133, and 2-AG, also failed to induce actin polymerisation in the day 5 and 12 PBLs, one of the key steps in cell migration (Ridley *et al.*, 2003), although 2-AG has been shown to stimulate this response in HL-60 cells differentiated into macrophage-like cells (Gokoh *et al.*, 2005b). This is consistent with their inability to induce chemotaxis in PBLs. Thus it may be that T lymphocytes do not migrate in response to any cannabinoids, although a wider selection of ligands would have to be tested to confirm this.

4.6.3.2 2-AG and JWH-133 inhibit CXCL12-induced migration

In addition to inducing basal migration, cannabinoids have also been suggested to modulate ligand-induced migration in several cell types, including some leukocytes. For instance, CP55,940 inhibited fMLP-induced migration of rat peritoneal macrophages (Sacerdote *et al.*, 2000) and JWH-133 and AEA inhibited CXCL12-induced motility of naïve human $CD8^+$ T lymphocytes (Joseph *et al.*, 2004). The data from the current study corroborates these findings by showing that JWH-133 also inhibits CXCL12-induced chemotaxis of a mainly $CD4^+$ population of activated T lymphocytes, although further investigation is

need to confirm whether it is the $CD4^+$ or $CD8^+$ T lymphocytes in the population responding. In addition, it also shows that 2-AG exerts the same response and that chemotaxis, rather than chemokinesis, is specifically affected. During the preparation of this manuscript another study was published which again showed that cannabinoids inhibited CXCL12-induced migration, this time of Jurkats and naïve $CD4^+$ and $CD8^+$ T lymphocytes (Ghosh *et al.*, 2006). Thus, whilst cannabinoids appear not to act as chemoattractants for T lymphocyte migration, they are clearly capable of inhibiting CXCL12-induced migration. It remains to be determined whether they will also affect migration of T lymphocytes toward other chemoattractants.

4.6.3.2.1 How could the cannabinoids be mediating this effect?

Joseph *et al* (2004) concluded that cannabinoid inhibition of CXCL12-induced migration was CB_2R-dependent as the response to AEA was mimicked by the CB_2R-selective agonist, JWH-133, but not a CB_1R-selective agonist, docosatetraenyltheanolamide. Similarly, Ghosh *et al* (2006) used the CB_2R-selective agonist JWH-015 to show that the CB_2R was involved, although they used high micromolar concentrations of this potent compound, which brings into question its selectivity. The findings from the current study also suggest a role for the CB_2R in this response as JWH-133 mimicked the effect of 2-AG. However, given that JWH-133 is CB_2R-selective rather than specific and at 100nM may also activate the CB_1R, further work needs to be carried out in order to verify the involvement of the CB_2R in this response. Interestingly, 2-AG and JWH-133 inhibited CXCL12-induced migration of both day 5 and day 12 PBLs. This suggests that if the response is CB_2R-mediated that only low receptor numbers may be necessary for cannabinoids to exert their actions, as discussed earlier with respect to ERK phosphorylation. As well as the CB_1R and CB_2R, additional cannabinoid receptors have been reported to exist such as the abn-cannabidiol sensitive receptor (Begg *et al.*, 2005). Activation of the abn-cannabidiol sensitive receptor has been reported to stimulate migration of both human umbilical vein endothelial cells (Ming Mo *et al.*, 2004) and murine microglial cells (Walter *et al.*, 2003). Whether it may be involved in the cannabinoid-mediated effects observed in the current study is unclear as reports

on whether 2-AG can activate abn-cannabidiol sensitive receptor have been mixed (Walter *et al.*, 2003, Begg *et al.*, 2005). Other cannabinoid targets include the orphan receptor GPR55 (Baker *et al.*, 2006). However, as mentioned earlier, JWH-133 has been reported not to activate GPR55 (Baker *et al.*, 2006) and is therefore unlikely to be involved in the effects on CXCL12-induced migration reported here.

Whether 2-AG affected CXCL12-induced actin polymerisation was briefly investigated as actin polymerisation. However, the inhibitory effect of 2-AG was mimicked by the vehicle control, suggesting that this effect was likely to be an experimental artefact. Thus, the likely mechanisms by which cannabinoids may interfere with chemokine driven directed cell migration are unclear at present. Cannabinoids have been widely shown to inhibit cAMP production (Demuth & Molleman, 2006) and it has been suggested that it is this inhibition of cAMP signalling that may be involved in their downregulation of chemokine-induced migration (Joseph *et al.*, 2004). However, given that 2-AG actually increased forskolin-induced cAMP production in day 5 PBLs and had no effect on basal cAMP production, this is unlikely to be the case. In addition, JWH-133 had no effect on forskolin-induced cAMP production but, like 2-AG, inhibited CXCL12-induced chemotaxis, further indicating that the effects on cAMP are not involved.

CXCL12 binds the receptor CXCR4 and another possibility is that cannabinoid receptor-CXCR4 dimers or oligomers form in the presence of cannabinoids and that this alters the signalling induced by CXCL12. There is evidence to suggest that the CB_1R can exist as homodimers (Wager-Miller *et al.*, 2002, Mackie, 2005, Xu *et al.*, 2005) as well as form heterodimers with GPCRs (Hilairet *et al.*, 2003, Kearn *et al.*, 2005, Wright *et al.*, 2005, Rios *et al.*, 2006). CB_2R homodimers have also been suggested to exist (Filppula *et al.*, 2004) and CXCR4 has also been shown to homo and heterodimerise (Percherancier *et al.*, 2005). It is also well established that ligand binding can regulate dimer formation and that heterodimer formation can alter the downstream signalling of the component receptors (Terrillon & Bouvier, 2004). Another possibility is that cannabinoids are causing heterologous desensitisation of CXCR4. It has previously been

demonstrated that activation of receptors such as opioid receptors on the surface of leukocytes can inhibit chemokine receptor function through this mechanism (Steele *et al.*, 2002). Cannabinoid treatment could also be affecting CXCR4 expression. One study has shown that *in vitro* Δ^9-THC can inhibit TNFα-induced increases in CCR2 mRNA expression in mouse splenocytes although the authors failed to mention how long they stimulated with Δ^9-THC (Steffens *et al.*, 2005b). It was recently reported that cannabinoids had no effect on CXCR4 expression in Jurkats over one hour (Ghosh *et al.*, 2006). Thus, it seems unlikely that cannabinoids would be affecting CXCR4 expression in this study. However, an additional receptor for CXCL12 was recently identified, RDC1, which is also expressed in T lymphocytes, especially $CD4^+$ T lymphocytes, and is involved in CXCL12-induced migration of T lymphocytes (Balabanian *et al.*, 2005, Infantino *et al.*, 2006). Thus, 2-AG and JWH-133 could also be affecting the ability of CXCL12 to induce migration through this receptor.

Cannabinoids may also be competing for or regulating pathways involved in cell migration. For example, the CB_1R has been reported to sequester Gi/o-proteins, making them unavailable to other GPCRs (Vasquez & Lewis, 1999). CXCR4 is Gi/o-protein coupled and thus cannabinoids, especially if the CB_2R shares this ability, could be inhibiting CXCR4 function from a point high up in the signalling cascade. However, given that 2-AG appears to inhibit CXCL12-induced chemotaxis but not chemokinesis this is unlikely.

One recently published study has investigated the effects of cannabinoids on the activity of Rho-family GTPases, which are involved in establishing cell polarisation (Fukata *et al.*, 2003) and found that these were differentially regulated by CB_2R activation (Kurihara *et al.*, 2006). Furthermore, they also demonstrated that HL-60 cells differentiated into neutrophil-like cells stimulated with 2-AG extended one or more pseudopods in different directions rather than developing front/rear polarity in response to fMLP as normally exhibited by chemotaxing leukocytes (Kurihara *et al.*, 2006). This data could explain why 2-AG affected the chemotaxis, but not chemokinesis, of day 5 PBLs to CXCL12 if 2-AG is affecting the ability of cells to migrate in a particular direction.

The effects of cannabinoids on many of the signalling molecules involved in migration have not yet been studied. However, they have, for example, been shown to activate PI3K, a key enzyme involved in several aspects of migration, in several different cell types (Bouaboula *et al.*, 1997, Galve-Roperh *et al.*, 2002). This activation may not be sufficient to elicit migratory responses alone but may lead to non-polarised accumulation of the 3' phosphorylated PI lipid products of PI3K which interfere with cellular navigation mechanisms. Alternatively, cannabinoid-activated PI3K may compete for PI lipid substrate available to chemokine-stimulated PI3Ks which again disrupts polarised PI lipid accumulation. Cannabinoids can also activate ERK1/2 and PKC as shown in several studies, including this one, and ERK1/2 can influence cell motility (Klemke *et al.*, 1997, Huang *et al.*, 2004, Larsson, 2006). However, this is unlikely to be involved in the inhibition of CXCL12-induced migration as Ghosh *et al* (2006) showed that JWH-015 inhibition of CXCL12-induced migration of Jurkats was unaffected by PD98059.

Furthermore, *in vivo*, cannabinoids may also affect cell adhesion (Gokoh *et al.*, 2005a, Grimaldi *et al.*, 2006) and/or production of chemokines (Jbilo *et al.*, 1999, Smith *et al.*, 2001). For instance, it was recently reported that 2-AG enhances the adhesion of HL-60 cells differentiated into macrophage-like cells to fibronectin in a CB_2R-dependent manner (Gokoh *et al.*, 2005a). Cannabinoids have been shown to both increase and decrease the production of chemokines. For example, in mice treated with thioglycollate broth to induce peritonitis it was found that two synthetic cannabinoids HU210 and WIN55,212-2 blocked the migration of neutrophils into the peritoneal cavity and that this effect was caused by a delay in the production of chemoattractants CXCL1 and CXCL2/3 (Smith *et al.*, 2001). On the other hand, CP55,940, a synthetic cannabinoid, was shown to increase CXCL8, CCL2 and CCL4 mRNA and protein production in wild type and CB_2R overexpressing HL-60 cells (Jbilo *et al.*, 1999, Derocq *et al.*, 2000). Finally, they may also affect migration velocity as JWH-015 and 2-AG have been reported to reduce fMLP-stimulated migration velocity of human neutrophils (Kurihara *et al.*, 2006).

4.6.4 Cannabinoids appear to modulate proliferation of activated T lymphocytes

In this part of the study JWH-133 was shown to increase IL-2-induced day 5 SEB-activated PBL numbers when this was measured using cell counting. The majority of studies have shown that cannabinoids act to decrease lymphocyte cell number. For instance, murine splenocytes treated with Δ^9-THC have a reduced proliferative response to mitogens (Pross *et al.*, 1990, Schatz *et al.*, 1993, McKallip *et al.*, 2002b), Δ^9-THC also suppresses human T lymphocyte proliferation stimulated by allogeneic dendritic cells (Yuan *et al.*, 2002), AEA inhibited mitogen-induced proliferation of human PBMCs (Schwarz *et al.*, 1994) and NADA inhibited proliferation of activated T lymphocytes (Sancho *et al.*, 2004). Apoptosis has been suggested to account for at least some of the cannabinoid-induced suppression of cell number detected (Schwarz *et al.*, 1994, Flygare *et al.*, 2005). However, some studies have also shown that cannabinoids can increase lymphocyte cell number. For example Δ^9-THC, CP55,940 and WIN55,212-2 increased proliferation of activated human B lymphocytes in a PTX-sensitive manner (Derocq *et al.*, 1995) and 2-AG stimulated the proliferation of a M-CSF-dependent rat microglial ell line in the presence of M-CSF (Carrier *et al.*, 2004). One study has suggested that the differential effects of cannabinoids on lymphocyte proliferation may be concentration-dependent (Luo *et al.*, 1992). The authors showed that Δ^9-THC increased PHA or concanavalin A-stimulated proliferation of human peripheral blood lymphocytes at low concentrations but inhibited proliferation at high concentrations (Luo *et al.*, 1992). Although admittedly the concentration range of JWH-133 tested in this first set of experiments was not very extensive a concentration-dependent effect was not noted. However, the use of JWH-133 did suggest that this response was CB_2R-mediated, which would be consistent with reports that cannabinoid-stimulated proliferation of activated human B lymphocytes (Derocq *et al.*, 1995) and rat microglial cells (Carrier *et al.*, 2004) was CB_2R-dependent.

The effect of JWH-133 on IL-2-induced day 5 PBL proliferation was not mimicked in day 11 PBLs. In these cells JWH-133 had no effect on IL-2-induced proliferation. As the increase in IL-2-induced proliferation appears to be

CB_2R-dependent and given that day 11 SEB-activated PBLs express less CB_2R protein than day 5 PBLs it is possible that the lack of response in day 11 PBLs is related to this lack of receptors. This is in stark contrast to results discussed earlier which showed that in day 12 PBLs cannabinoids still coupled to ERK1/2 phosphorylation and inhibited CXCL12-induced chemotaxis. This could suggest that a higher receptor occupancy is required to affect proliferation than other functions. However, the day 11 PBLs generally proliferated less, even in response to IL-2, and therefore this lack of response may simply be a reflection of a general physiological phenomenon.

Counting cells using the Coulter counter is a very low throughput way of investigating cell proliferation so XTT assays were used to further investigate the effects of cannabinoids on IL-2-induced PBL proliferation. The assay works on the principle that XTT, which is a tetrazolium salt, will be converted to an orange formazan dye only in metabolically active cells. The colour change can be measured and the absorbance will reflect the number of cells present in the wells. Although actual cell numbers cannot be determined using this assay it still allows a comparison to be made between different experimental conditions. Again, both the day 5 and day 11 PBLs responded to IL-2 in a concentration-dependent manner. The effects of JWH-133, ACPA, a CB_1R-selective agonist and met-AEA, a stable AEA analogue, were variable between experiments resulting in large error bars. There was a trend though for all three cannabinoids to increase IL-2-induced day 5 PBLs proliferation. The increases were only modest, however, and were not statistically significant compared to vehicle due to the large amount of variability. The response to JWH-133 again suggests the involvement of the CB_2R. Although ACPA was used as a CB_1R-selective agonist, it only increased proliferation at 10μM, when it would no longer discriminate between the two cannabinoid receptors (Hillard *et al.*, 1999). In fact at the lower concentrations when ACPA is selective for the CB_1R ACPA appears to be inhibiting proliferation slightly. The increase in IL-2-induced proliferation stimulated by the cannabinoids in day 11 PBLs was even smaller than in day 5 PBLs. Again, there was a large amount of variability between experiments.

Although the XTT assays do support the data from the cell counts to a certain extent they were not as helpful in determining the effects of cannabinoids on proliferation as hoped. The main problem encountered was the variability between experiments which showed that in some experiments the cannabinoids almost inhibited proliferation of, for instance, day 5 PBLs in response to IL-2, whereas in others they enhanced proliferation. This variability may reflect that XTTs are not as accurate a way of determining cell numbers or it could reflect a true variability in the response of cells to cannabinoids. It has been shown that the activation status of murine splenocytes greatly influences the ability of cannabinol to stimulate IL-2 production (Jan & Kaminski, 2001). Cannabinol enhanced IL-2 production in suboptimally activated cells but suppressed IL-2 secretion in optimally activated cells. So subtle differences between individual sets of PBLs could result in different effects of the cannabinoids such as regulating the extent of proliferation they induce. This would suggest that the cannabinoids have the ability to respond very well to the exact cellular environment and/or status. This is also reflected in the fact that lymphocyte proliferation was shown to be differentially affected by Δ^9-THC depending on mitogen used (Pross *et al.*, 1987, Pross *et al.*, 1992) and cell density (Pross *et al.*, 1987, Schatz *et al.*, 1993). Further experiments would need to be carried out to assess whether experimental conditions such as cell density affect the proliferative response induced by JWH-133 in the day 5 PBLs.

4.6.4.1 How could JWH-133 be mediating this effect?

Cannabinoids couple to several pathways that could be linked to the increase in IL-2-induced proliferation of day 5 PBLs. For instance, cannabinoids have been shown to couple to PI3K activation (Bouaboula *et al.*, 1997, Galve-Roperh *et al.*, 2002) and PI3K has in turn been shown to be important in the regulation of T lymphocyte proliferation (Breslin *et al.*, 2005). Similarly, cannabinoids can couple to ERK activation in these cells and the ERK signalling cascade is linked to the control of proliferation (Stork & Schmitt, 2002). As day 12 PBLs also coupled to ERK1/2 phosphorylation in this study but JWH-133 failed to enhance proliferation as shown by cell counting, other signalling pathways would clearly also be involved in this response in day 5 PBLs. Cannabinoids have also been

shown to modulate energy metabolism in a number of cell types (Guzman & Sanchez, 1999). For example, Δ^9-THC, at nanomolar concentrations, enhanced glucose oxidation and phospholipid synthesis from glucose in murine splenocytes (Sanchez *et al.*, 1997). The ability to enhance energy metabolism may play some part in the pro-proliferative actions of cannabinoids.

4.7 Conclusions

In this part of the study I have shown that AEA and 2-AG couple to ERK1/2 phosphorylation in both activated PBLs and Jurkats. Although the responses are PTX-sensitive suggesting that they are receptor mediated the involvement of the CB_2R and/or modified CB_1R could not be specifically determined due to experimental difficulties. However, as the response is mimicked by JWH-133 it is a strong indication that the CB_2R is involved. Evidence from naive T lymphocytes, which express only the modified CB_1R, suggests that this receptor may not be functional although this needs to be confirmed by additional experiments. ERK is coupled to several cellular functions and thus cannabinoids could influence a number of activated T lymphocyte functions by activating ERK1/2, including proliferation (Stork & Schmitt, 2002) and migration (Huang *et al.*, 2004) as discussed.

2-AG was shown to enhance forskolin-induced cAMP production in day 5 PBLs. Again, there are many downstream effectors of cAMP and therefore any change in cAMP production has the potential to affect a myriad of cellular functions. For instance, cAMP has been shown to both activate and inhibit cell proliferation (Stork & Schmitt, 2002) and can also influence cell migration and cytoskeletal organisation (Howe, 2004). Thus, by elevating levels of cAMP, 2-AG could have a number of effects on activated T lymphocyte function. Further studies would need to be done to elucidate its exact role.

In this part of the study it was also determined that although neither 2-AG nor JWH-133 act as chemoattractants for T lymphocytes these cannabinoids do inhibit CXCL12-induced chemotaxis. The implications of this data are that cannabinoids may inhibit infiltration of activated T lymphocytes into sites of

infection and may therefore be one of their immunosuppressive actions. In contrast, I have also determined that JWH-133 can increase IL-2-induced proliferation of day 5, but not day 11, PBLs in a CB_2R-dependent manner. At first glance this suggests that the cannabinoids may be pro-inflammatory. However, it is possible that the response may vary depending on the cellular environment and activation status, indicating that cannabinoids may be immunomodulatory rather than strictly pro-inflammatory or immunosuppressive.

Chapter 5: Final Discussion and Future Work

5.1 Final Discussion

During the course of this study it has been shown that the 83kD protein detected by the Cayman Chemicals CB_1R antibody in the colonic epithelial cell line HT-29s, leukaemic T lymphocyte cell lines, CEMs and Jurkats, naïve purified primary human T lymphocytes and PBLs appears to be a modified form of the receptor. However, at present it remains unclear how it is modified although data indicates that it is not *N*-glycosylated. It is also unknown whether the protein represents a functional receptor or not. Immunoblotting of HT-29 fractions suggested that it can be present at the membrane, however data indicates that it is not activated by the cannabinoids. For instance, neither AEA nor 2-AG induced ERK1/2 or PKB phosphorylation in naïve purified T lymphocytes, cells which were shown to express only this modified 83kD form of the CB_1R and no CB_2R. Furthermore, ACPA, which is believed to be a CB_1R-selective agonist, although it has also been described as a broad cannabinoid agonist in one study (Franklin & Stella, 2003), did not enhance proliferation except at very high concentrations, when it would certainly also bind the CB_2R. Thus at present is appears that the 83kD form of the CB_1R detected in PBLs may not represent a functional receptor. However, further experiments are required to confirm this.

Although the cannabinoids were originally believed to be immunosuppressive, impairing cell-mediated and humoral immunity and cellular defences against infectious agents such as viruses, more recent studies have shown that they can exert both pro and anti-inflammatory actions (Cabral & Dove Pettit, 1998, Croxford & Yamamura, 2005). Thus, they are now generally referred to as immunomodulatory. Given that they can exert a wide range of, often contradictory, actions it has been difficult to determine whether cannabinoids could be exploited therapeutically for immune-mediated diseases. Nonetheless, this avenue of research continues as studies have shown that cannabinoids may be beneficial in diseases such as multiple sclerosis, rheumatoid arthritis, allergic asthma and IBD (Croxford & Yamamura, 2005, Klein, 2005).

One of the main difficulties in determining the usefulness of cannabinoids as therapeutic agents in the field of inflammatory diseases is that many studies investigating the effects of cannabinoids on immune cell function have been carried out on non-human primary cells such as murine or rat cells or transformed cell lines. Although these are certainly useful they also have their limitations as it has been shown that there are significant differences between mouse and human immunology in general (Mestas & Hughes, 2004) and there are differences between the cannabinoid receptor orthologues. For instance, the rat CB_2R has a longer C-terminus compared to the human and mouse CB_2R which may affect receptor desensitisation and internalisation following activation (Brown *et al.*, 2002) and there are also pharmacological differences between the human and rat CB_2Rs such as receptor selectivity of various cannabinoids (Mukherjee *et al.*, 2004). The inactivation of 2-AG by COX-2 also appears to occur at different rates in human and rat plasma (Kozak *et al.*, 2001). Furthermore, cannabinoids may have different effects on transformed and non-transformed cells. For instance, they induce apoptosis of transformed neural cells but may protect non-transformed neurons from insults such as ischaemia and oxidative damage (Guzman *et al.*, 2002). In addition, the Jurkats used in this current study express a modified CB_1R was detected, although there are several reports stating that Jurkats express no (Bouaboula *et al.*, 1993, Schatz *et al.*, 1997, McKallip *et al.*, 2002a) or very little (Daaka *et al.*, 1996) CB_1R mRNA and no protein (Daaka *et al.*, 1996). Also, it was shown that FAAH was resolved as a doublet in Jurkats and CEMs but not in PBLs, suggesting it may be differently post-translationally modified in primary and transformed cells. All in all these examples show that as well as there being potential differences between clones of a particular cell line, differences in the cannabinoid system exist between primary cells and cell lines and that it is important to use human cells if the data is to be extrapolated to potential therapeutic use. For these reasons the majority of the work in this study was carried out in primary human cells.

The question is then whether the data collected in this study indicates that cannabinoids exert immunostimulatory or immunosuppressive effects on activated T lymphocytes. The results suggest once again that the cannabinoid are

mainly modulatory and have the potential to exert pro and anti-inflammatory effects depending on the function studied.

Although the results were preliminary, and further work does need to be carried out to confirm this finding, 2-AG enhanced forskolin-induced cAMP production in day 5 PBLs. Given that this was not mimicked by the CB_2R-selective agonist JWH-133 and the modified CB_1R expressed on these cells appears not to be functional, this suggests that 2-AG is acting via a cannabinoid receptor-independent mechanism. It was long believed that cAMP-dependent signalling pathways were involved in downregulating the immune response but this perception was largely based on studies that used high (>100μM) concentrations of cAMP analogues. More recently it has become apparent that cAMP signalling pathways can also be involved in immunostimulatory actions as shown in studies using lower (<100μM) concentrations of cAMP analogues (Kaminski, 1998). Thus, by elevating cAMP production 2-AG could be either stimulating or inhibiting leukocyte functions and this may depend particular end point studied.

It has previously been shown that bacterial activation of human PBLs decreases levels of FAAH and increases levels of endocannabinoids (Maccarrone *et al.*, 2001). In this study it has been shown that T lymphocyte activation by the superantigen SEB increases the expression of the CB_2R protein. Thus, we can envisage a scenario were naïve T lymphocytes are activated and upregulate expression of the both endocannabinoids and the CB_2R. Given that cannabinoids inhibit CXCL12-induced chemotaxis of activated T lymphocytes, such as the day 5 PBLs, most likely via the CB_2R, the implications of this data are that cannabinoids may inhibit infiltration of activated T lymphocytes into sites of infection and therefore be immunosuppressive. This correlates with a study showing that infiltrating $CD4^+$ T lymphocyte numbers were suppressed in mouse spinal cord by several synthetic cannabinoids, including JWH-015, a CB_2R-selective agonist, in a murine model of multiple sclerosis (Arevalo-Martin *et al.*, 2003). The same response is detected in both day 5 and 12 PBLs, even when CB_2R expression is low. This could reflect a low receptor occupancy requirement as discussed earlier but does raise the question of why the receptor is

upregulated and then downregulated if this is not to affect how or the extent to which T lymphocytes respond to cannabinoids.

Notably, data collected suggests that whilst cannabinoids increase IL-2-induced proliferation of day 5 PBLs, day 11 PBL proliferation is unaffected. Thus it appears that the changes in CB_2R receptor expression affects some but not all T lymphocyte functions. Further studies would need to be carried out to determine which other T lymphocyte functions are affected by the changes in receptor expression. That JWH-133 stimulated IL-2-induced proliferation of day 5 PBLs, which is an immunostimulatory response, was unexpected. Other studies have shown that cannabinoids inhibit lymphocyte proliferation (Pross *et al.*, 1990, Schatz *et al.*, 1993, McKallip *et al.*, 2002b, Yuan *et al.*, 2002) and the current and others (Joseph *et al.*, 2004, Ghosh *et al.*, 2006) have shown that cannabinoids inhibit CXCL12-induced migration T lymphocytes, an immunosuppressive response. Thus, cannabinoids may, during this early phase of the immune response when CB_2R expression is high, have a regulatory role and depending on the microenvironment either inhibit infiltration into sites of inflammation or stimulate proliferation. In contrast, we can envisage that later in the immune response, when CB_2R expression is lower and they no longer affect proliferation, cannabinoids continue to inhibit infiltration into sites of inflammation, which may play a role in the resolution phase of inflammation. Indeed, in the context of IBD, which was part of the original rationale or focus behind the project, these findings may be very important. It was published recently that levels of AEA are upregulated in inflamed colon of IBD patients as well as in animal models of IBD and that increasing levels of AEA resulted in ameliorated inflammatory scores in those animal models (D'Argenio *et al.*, 2006). Given the important role that T lymphocytes play in IBD (MacDonald & Monteleone, 2005), inhibition of their infiltration into sites of inflammation may represent one of the mechanisms by which AEA exerts this control on inflammation in IBD.

5.2 Future Work

5.2.1 Further exploration into the nature of the 83kD protein detected

One of main unresolved issues arising from this work is the identity of the 83kD CB_1R band detected. Although the data generated indicates that it is indeed a form of the receptor, how it has been modified and whether it is functional as a cannabinoid receptor remains unclear. Verifying whether the Jurkats used in this project express CB_1R mRNA would help determine once and for all whether these Jurkats do express the CB_1R and hence whether the 83kD protein detected is a CB_1R. Immunoblotting of WCLs prepared from HT-29s in which the CB_1R has been silenced using siRNA technology would also confirm whether or not the 83kD band detected represents a CB_1R isoform. Recently an additional CB_1R antibody raised against a portion of the C-terminus of the receptor has become commercially available and would be a useful tool in determining whether the 83kD protein is detected purely because of the epitope against which it was raised or is a high molecular weight form of the receptor. Equally, ligand binding experiments on Jurkat or PBL cell membranes, using a CB_1R-selective agonist, would also help confirm whether or not the 83kD protein detected is a receptor. However, care would need to be taken in choosing such an agonist as ACPA, a commonly used compound, has recently been shown to be a general cannabinoid receptor agonist (Franklin & Stella, 2003). Selective agonists could also be used to determine whether the receptor expressed is functional and couples to downstream signalling events such as phosphorylation of ERK1/2. Prelimary data also indicated that in HT-29s, which express both the CB_1R and CB_2R, these two receptors dimerise. Given that cannabinoid receptor dimerisation may affect how we understand cannabinoid pharmacology in cells that express both receptors, this should be further explored. In particular, in relation to this project, it will be important to determine whether the 83kD protein and the CB_2R can dimerise in T lymphocytes.

5.2.2 Investigating the effects of additional T lymphocyte activators on receptor expression

The temporal variation in CB_2R protein expression following T lymphocyte activation is one of the main discoveries made during this study and given that this implies that cannabinoids may affect T lymphocyte function in different ways throughout the immune response, should be investigated further. A difference in the length of time of CB_2R protein upregulation was detected when SEB or CD3/CD28-coated beads were used as T lymphocyte activators. This work could be extended to other activators, such as PHA. Furthermore, day 7-8 SEB-activated PBLs, when CB_2R expression starts to be downregulated, could be incubated with CD3/CD28-coated beads to examine whether protein expression would again be upregulated. This would confirm that the upregulation is connected specifically to T lymphocyte activation.

5.2.3 Exploring the mechanisms by which cannabinoid inhibit CXCL12-induced migration

During the course of this study it was determined that 2-AG and JWH-133 inhibit CXCL12-induced migration of day 5 and 12 SEB-activated PBLs. As well as extending this data to additional cannabinoid agonists further work needs to be carried out to confirm the involvement of the CB_2R and to verify whether the effect can be repeated in purified naïve T lymphocytes. Given that they do not appear to express the CB_2R it is expected that CXCL12-induced migration will not be affected. The use of the CB_1R and CB_2R antagonists AM251 and AM630 proved problematic in the signalling experiments but may be more successful in these functional studies. Alternatively siRNA technology could be used to silence the CB_2R and water soluble cannabinoids are being synthesised which would hopefully eliminate problems with vehicle controls (Pertwee *et al.*, 2000, Martin *et al.*, 2006). Further work also needs to be carried out to determine the mechanism by which cannabinoids are eliciting this effect. For example the effects of cannabinoids on CXCL12-induced signalling could be examined and the study on cannabinoid effects on fMLP-induced polarization of neutrophils (Kurihara *et al.*, 2006) repeated in PBLs. Equally the experiments

carried out during this study should be repeated with other chemoattractants in order to determine whether the response is CXCL12-specific. It should also be investigated whether it is the $CD4^+$ or $CD8^+$ T lymphocytes, or both, that are responding.

5.2.4 Further investigation into the potentially pro-proliferative actions of cannabinoids on activated T lymphocytes

Finally, although data indicated that JWH-133 is capable of increasing day 5, but not day 11, PBL proliferation, additional experiments are required to verify this and extend the observation to additional agonists. The involvement of the receptors in this response also needs to be studied. In addition, it has been suggested that experimental factors such as cell density can affect cannabinoid responses on cell number (Pross *et al.*, 1987, Schatz *et al.*, 1993) and thus it would be important to determine whether this is true in activated T lymphocytes as this could affect *in vivo* responses. Again, it would also be interesting to determine whether it is the $CD4^+$ or $CD8^+$ T lymphocytes, or both, that are responding, especially as some differences in the responsiveness of particular T lymphocyte subsets to cannabinoids has previously been noted (Pross *et al.*, 1990).

Chapter 6: References

Alessi D, Cuenda A, Cohen P, Dudley D, Saltiel A. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *Journal of Biological Chemistry* 1995; **270**:27489-27494.

Ambrosi S, Ragni L, Ambrosini A, Paccamiccio L, Marianni P, Fiorini R, Bertoli E, Zolese G. On the importance of anandamide structural features for its interactions with DPPC bilayers: effects on PLA_2 activity. *Journal of Lipid Research* 2005; **46**:1953-1961.

Anai M, Shojima N, Katagiri H, Ogihara T, Sakoda H, Onishi Y, Ono H, Fujishiro M, Fukushima Y, Horike N, Viana A, Kikuchi M, Noguchi N, Takashashi S, Takata K, Oka Y, Uchijima Y, Kurihara H, Asano T. A novel protein kinase B (PKB)/AKT-binding protein enhances PKB kinase activity and regulates DNA synthesis. *Journal of Biological Chemistry* 2005; **280**:18525-18535.

Anday J, Mercier R. Gene ancestry of the cannabinoid receptor family. *Pharmacological Research* 2005; **52**:463-466.

Anderson D. Role of lipids in the MAPK signaling pathway. *Progress in Lipid Research* 2006; **45**:102-119.

Andersson H, D'Antona A, Kendall D, Von Heijne G, Chin C. Membrane assembly of the cannabinoid receptor 1: impact of a long N-terminal tail. *Molecular Pharmacology* 2003; **64**:570-577.

Arevalo-Martin A, Vela J, Molina-Holgado E, Borrell J, Guaza C. Therapeutic action of cannabinoids in a murine model of multiple sclerosis. *Journal of Neuroscience* 2003; **23**:2511-2516.

Astoul E, Cantrell D, Edmunds C, Ward S. PI 3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. *Trends in Immunology* 2001; **22**:490-496.

Baker D, Pryce G, Davies W, Hiley CR. In silico patent searching reveals a new cannabinoid receptor. *Trends in Pharmacological Sciences* 2006; **27**:1-4.

Balabanian K, Lagane B, Infantino S, Chow K, Harriagne J, Moepps B, Renzana-Seisdedos F, Thelen M, Bacchelerie F. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *Journal of Biological Chemistry* 2005; **280**:35760-35766.

Barann M, Molderings G, Bruss M, Bonisch H, Urban B, Gothert M. Direct inhibition by cannabinoids of human $5\text{-}HT_{3A}$ receptors: probable involvement of an allosteric modulatory site. *British Journal of Pharmacology* 2002; **137**:589-596.

Bari M, Paradisi A, Pasquariello N, Maccarrone M. Cholesterol-dependent modulation of type 1 cannabinoid receptors in nerve cells. *Journal of Neuroscience Research* 2005a; **81**:275-283.

Bari M, Battista N, Fezza F, Finazzi-Agro A, Maccarrone M. Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells: implications for anandamide-induced apoptosis. *Journal of Biological Chemistry* 2005b; **280**:12212-12220.

Bayewitch M, Vidor-Reiss T, Levy R, Barg J, Mechoulam R, Vogel Z. The peripheral cannabinoid receptor: adenylate cyclase inhibition and G-protein coupling. *FEBS Letters* 1995; **375**:143-147.

Begg M, Pacher P, Batkai S, Osei-Hyiaman D, Offertaler L, Mo F, Lie J, Kunos G. Evidence for novel cannabinoid receptors. *Pharmacology & Therapeutics* 2005; **106**:133-145.

Berdyshev E. Cannabinoid receptors and the regulation of immune response. *Chemistry & Physics of Lipids* 2000; **108**:169-190.

Berdyshev E, Boichot E, Germain N, Allain N, Anger JP, Lagente V. Influence of fatty acid ethanolamides and Δ^9-tetrahydrocannabinol on cytokine and arachidonate release by mononuclear cells. *European Journal of Pharmacology* 1997; **330**:231-240.

Berdyshev E, Schmid P, Krebsbach R, Krebsbach R, Hillard C, Huang C, Chen N, Dong Z, Schmid. Cannabinoid-receptor-independent cell signalling by N-acylethanolamines. *Biochemical Journal* 2001; **360**:67-75.

Bisogno T, Ligresti A, Di Marzo V. The endocannabinoid signalling system: biochemical aspects. *Pharmacology, Biochemistry and Behaviour* 2005; **81**:224-238.

Bisogno T, Melck D, Bobrov M, Gretskaya N, Bezuglov V, De Petrocellis L, Di Marzo V. *N*-acyl-dopamines: novel synthetic CB1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity *in vitro* and *in vivo*. *Biochemical Journal* 2000; **351**:817-824.

Bisogno T, Maccarrone M, De Petrocellis L, Jarrahian A, Finazzi-Agro A, Hillard C, Di Marzo V. The uptake by cells of 2-arachidonoylglycerol, an endogenous agonist of cannabinoid receptors. *European Journal of Biochemistry* 2001; **268**:1982-1989.

Blanchard D, Newton C, Klein T, Stewart, Friedman H. *In vitro* and *in vivo* suppressive effects of Δ^9-tetrahydrocannabinol on interferon production by murine spleen cells. *International Journal of Immunopharmacology* 1986; **8**:819-824.

Bleul C, Fuhlbrigge R, Casasnovas J, Aiuti A, Springer T. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *Journal of Experimental Medicine* 1996; **184**:1101-1109.

Bloom A, Edgemond W, Moldvan J. Nonclassical and endogenous cannabinoids: Effects on the ordering of brain membranes. *Neurochemical Research* 1997; **22**:563-568.

Bonhaus D, Chang L, Kwan J, Martin G. Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *Journal of Pharmacology and Experimental Therapeutics* 1998; **287**:884-888.

Bos J. Epac: a new cAMP target and new avenues in cAMP research. *Nature Reviews in Molecular Cell Biology* 2003; **4**:733-738.

Bouaboula M, Bourrie B, Rinaldi-Carmona M, Shire D, Le Fur G, Casellas P. Stimulation of cannabinoid receptor CB1 induces krox-24 expression in human astrocytoma cells. *Journal of Biological Chemistry* 1995a; **270**:13973-13980.

Bouaboula M, Hilairet S, Marchand J, Fajas L, Le FG, Casellas P. Anandamide induced PPARγ transcriptional activation and 3T3-L1 preadipocyte differentiation. *European Journal of Pharmacology* 2005; **517**:174-181.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, Maffrand J, Le Fur G, Casellas P. A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *Journal of Biological Chemistry* 1997; **272**:22330-22339.

Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G, Casellas P. Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochemical Journal* 1995b; **312**:637-641.

Bouaboula M, Poinot-Chazel C, Marchand J, Canat X, Bourrie B, Rinaldi-Carmona M, Calandra B, Le Fur G, Casellas P. Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *European Journal of Biochemistry* 1996; **237**:704-711.

Bouaboula M, Rinaldi M, Carayon P, Carillon C, Delpech B, Shire D, Le Fur G, Casella P. Cannabinoid-receptor expression in human leukocytes. *European Journal of Biochemistry* 1993; **214**:173-180.

Bouaboula M, Dussossoy D, Casellas P. Regulation of peripheral cannabinoid receptor CB2 phosphorylation by the inverse agonist SR144528. Implications for receptor biological responses. *Journal of Biological Chemistry* 1999; **274**:20397-20405.

Bradshaw H, Walker J. The expanding field of cannabimimetic and related lipid mediators. *British Journal of Pharmacology* 2005; **144**:459-465.

Breslin E, White P, Shore A, Clement M, Brennan P. LY294002 and rapamycin co-operate to inhibit T-cell proliferation. *British Journal of Pharmacology* 2005; **144**:791-800.

Brock C, Schaefer M, Reusch H, Czupalla C, Michalke M, Spicher K, Schultz G, Nurnberg B. Roles of Gβγ in membrane recruitment and activation of p110γ/p101 phosphoinositide 3-kinase-γ. *Journal of Cell Biology* 2003; **160**:89-99.

Brose N, Rosenmund C. Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *Journal of Cell Science* 2002; **115**:4399-4411.

Brown S, Wager-Miller J, Mackie K. Cloning and molecular characterization of the rat CB2 cannabinoid receptor. *Biochimica et Biophysica Acta-Gene Structure and Expression* 2002; **1576**:255-264.

Buckley N, McCoy K, Mezey E, Bonner T, Zimmer A, Felder C, Glass M, Zimmer A. Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB2 receptor. *European Journal of Pharmacology* 2000; **396**:141-149.

Burstein S. PPARγ: A nuclear receptor with affinity for cannabinoids. *Life Sciences* 2005; **77**:1674-1684.

Cabral G, Dove Pettit D. Drugs and immunity: cannabinoids and their role in decreased resistance to infectious disease. *Journal of Neuroimmunology* 1998; **83**:116-123.

Campbell K, Perkins N. Regulation of NFκB function. *Biochemical Society Symposium* 2006;**73**: 165-180.

Carayon P, Marchand J, Dussossoy D, Derocq J, Jbilo O, Bord A, Bouaboula M, Galiegue S, Mondiere P, Penarier G, Le Fur G, Defrance T, Casellas P. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. *Blood* 1998; **92**:3605-3615.

Carding S, Egan P. γδ T cells: functional plasticity and heterogeneity. *Nature Reviews in Immunology* 2002; **2**:336-345.

Carlini E. The good and the bad effects of (-) trans-Δ^9-tetrahydrocannabinol (Δ^9-THC) on humans. *Toxicon* 2004; **44**:461-467.

Carlisle S, Marciano-Cabral F, Staab A, Ludwick C, Cabral G. Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *International Immunopharmacology* 2002; **2**:69-82.

Carrier E, Kearn C, Barkmeier A, Breese N, Yang W, Nithipatikom K, Pfister S, Campbell W, Hillard C. Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. *Molecular Pharmacology* 2004; **65**:999-1007.

Casu M, Porcella A, Ruiu S, Saba P, Marchese G, Carai M, Reali R, Gessa G, Pani L. Differential distribution of functional cannabinoid CB1 receptors in the mouse gastroenteric tract. *European Journal of Pharmacology* 2003; **459**:97-105.

Caterina M, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. *Annual Reviews in Neuroscience* 2001; **24**:487-517.

Chodniewicz D, Klemke R. Guiding cell migration through directed extension and stabilization of pseudopodia. *Experimental Cell Research* 2004; **301**:31-37.

Chuchawankul S, Shima M, Buckley N, Hartmann C, McCoy K. Role of cannabinoid receptors in inhibiting macrophage costimulatory activity. *International Immunopharmacology* 2004; **4**:265-278.

Condie R, Herring A, Koh W, Lee M, Kaminski N. Cannabinoid inhibition of adenylate cyclase-mediated signal transduction and interleukin 2 (IL-2) expression in the murine T-cell line, EL4.IL-2. *Journal of Biological Chemistry* 1996; **271**:13175-13183.

Craib S, Ellington H, Pertwee R, Ross R. A possible role of lipoxygenase in the activation of vanilloid receptors by anandamide in the guinea-pig bronchus. *British Journal of Pharmacology* 2001; **134**:30-37.

Cravatt B, Lichtman A. The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chemistry & Physics of Lipids* 2002; **121**:135-148.

Croxford J, Yamamura T. Cannabinoids and the immune system: Potential for the treatment of inflammatory diseases? *Journal of Neuroimmunology* 2005; **166**:3-18.

D'Argenio G, Valenti M, Scaglione G, Cosenza V, Sorrentini I, Di Marzo V. Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. *FASEB Journal* 2006; **20**:568-570.

Daaka Y, Friedman H, Klein T. Cannabinoid receptor proteins are increased in Jurkat, human T-cell line after mitogen activation. *Journal of Pharmacology and Experimental Therapeutics* 1996; **276**:776-783.

Damle N, Klussman K, Leytze G, Linsley P. Proliferation of human T lymphocytes induced with superantigens is not dependent on costimulation by the CD28 counter-receptor B7. *Journal of Immunology* 1993; **150**:726-735.

Davies S, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* 2000; **351**:95-105.

Davis M, Ronesi J, Lovinger D. A predominant role for inhibition of the adenylate cyclase/protein kinase A pathway in ERK activation by cannabinoid receptor 1 in N1E-115 neuroblastoma cells. *Journal of Biological Chemistry* 2003; **278**:48973-48980.

Day T, Rakhshan F, Deutsch D, Barker E. Role of fatty acid amide hydrolase in the transport of the endogenous cannabinoid anandamide. *Molecular Pharmacology* 2001; **59**:1369-1375.

De Petrocellis L, Melck D, Ueda N, Maurelli S, Kurahashi Y, Yamamoto S, Marino G, Di Marzo V. Novel inhibitors of brain, neuronal, and basophilic anandamide amidohydrolase. *Biochemical and Biophysical Research Communications* 1997; **231**:82-88.

De Petrocellis L, Cascio M, Di Marzo V. The endocannabinoid system: a general view and latest additions. *British Journal of Pharmacology* 2004; **141**:765-774.

De Petrocellis L, Orlando P, Di Marzo V. Anandamide, an endogenous cannabinomimetic substance, modulates rat brain protein kinase C *in vitro*. *Biochemistry and Molecular Biology International* 1995; **36**:1127-1133.

Dedov V, Tran V, Duke C, Connor M, Christie M, Mandadi S, Roufogalis B. Gingerols: a novel class of vanilloid receptor (VR1) agonists. *British Journal of Pharmacology* 2002; **137**:793-798.

Demuth D, Molleman A. Cannabinoid signalling. *Life Sciences* 2006; **78**:549-563.

Derkinderen P, Ledent C, Parmentier M, Girault J. Cannabinoids activate p38 mitogen-activated protein kinases through CB1 receptors in hippocampus. *Journal of Neurochemistry* 2001a; **77**:957-960.

Derkinderen P, Toutant M, Burgaya F, Le Bert M, Siciliano J, de Franciscis V, Gelman M, Girault J. Regulation of a neuronal form of focal adhesion kinase by anandamide. *Science* 1996; **273**:1719-1722.

Derkinderen P, Toutant M, Kadare G, Ledent C, Parmentier M, Girault J. Dual role of Fyn in the regulation of FAK+6,7 by cannabinoids in hippocampus. *Journal of Biological Chemistry* 2001b; **276**:38289-38296.

Derkinderen P, Valjent E, Toutant M, Corvol J, Enslen H, Ledent C, Trzaskos J, Caboche J, Girault J. Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. *Journal of Neuroscience* 2003; **23**:2371-2382.

Derocq J, Segui M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Letters* 1995; **369**:177-182.

Derocq J, Bouaboula M, Marchand J, Rinaldi-Carmona M, Segui M, Casellas P. The endogenous cannabinoid anandamide is a lipid messenger activating cell growth via a cannabinoid receptor-independent pathway in hematopoietic cell lines. *FEBS Letters* 1998; **425**:419-425.

Derocq J, Jbilo O, Bouaboula M, Segui M, Clere C, Casellas P. Genomic and functional changes induced by the activation of the peripheral cannabinoid receptor CB2 in the promyelocytic cells HL-60. Possible involvement of the CB2 receptor in cell differentiation. *Journal of Biological Chemistry* 2000; **275**:15621-15628.

Deusch E, Kraft B, Nahlik G, Weigl L, Hohenegger M, Kress H. No evidence for direct modulatory effects of Δ^9-tetrahydrocannabinol on human polymorphonuclear leukocytes. *Journal of Neuroimmunology* 2003; **141**:99-103.

Deutsch D, Glaser S, Howell J, Kunz J, Puffengarber R, Hillard C, Abumrad N. The cellular uptake of anandamide is coupled to its breakdown by fatty acid amide hydrolase. *Journal of Biological Chemistry* 2001; **276**:6967-6973.

Deutsch D, Omeir R, Arreaza G, Salehani D, Prestwich G, Huang Z, Howlett A. Methyl arachidonyl fluorophosphonate: a potent irreversible inhibitor of anandamide amidase. *Biochemical Pharmacology* 1997; **53**:255-260.

Devane W, Dysarz F, Johnson M, Melvin L, Howlett A. Determination and characterization of a cannabinoid receptor in rat brain. *Molecular Pharmacology* 1988; **34**:605-613.

Devane W, Hanus L, Breuer A, Pertwee R, Stevenson L, Griffin G, Gibson D, Mandelbaum A, Etinger A., Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 1992; **258**:1946-1949.

Di Marzo V, Bifulco M, De Petrocellis L. The endocannabinoid system and its therapeutic exploitation. *Nature Reviews in Drug Discovery* 2004; **3**:771-784.

Di Marzo V, Matias I. Endocannabinoid control of food intake and energy balance. *Nature Neuroscience* 2005; **8**:585-589.

Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner J, Kunos G. Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. *European Journal of Biochemistry* 1999; **264**:258-267.

Diaz S, Specter S, Coffey R. Suppression of lymphocyte adenosine 3':5'-cyclic monophosphate (cAMP) by Δ^9-tetrahydrocannabinol. *International Journal of Immunopharmacology* 1993; **15**:523-532.

Dinh T, Carpenter D, Leslie F, Freund T, Katona I, Sensi S, Kathuria S, Piomelli D. Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proceedings of the National Academy of Sciences, USA* 2002; **99**:10819-10824.

Do Y, McKallip R, Nagarkatti M, Nagarkatti P. Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NFκB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. *Journal of Immunology* 2004; **173**:2373-2382.

Downer E, Fogarty M, Campbell V. Tetrahydrocannabinol-induced neurotoxicity depends on CB1 receptor-mediated c-Jun N-terminal kinase activation in cultured cortical neurons. *British Journal of Pharmacology* 2003; **140**:547-557.

Dremier S, Kopperud R, Doskeland S, Dumont J, Maenhaut C. Search for new cyclic AMP-binding proteins. *FEBS Letters* 2003; **546**:103-107.

Duncan M, Millns P, Smart D, Wright J, Kendall D, Ralevic V. Noladin ether, a putative endocannabinoid, attenuates sensory neurotransmission in the rat isolated mesenteric arterial bed via a non-CB1/CB2 Gi/o-linked receptor. *British Journal of Pharmacology* 2004; **142**:509-518.

Edgemond W, Hillard C, Falck J, Kearn C, Campbell W. Human platelets and polymorphonuclear leukocytes synthesize oxygenated derivatives of arachidonylethanolamide (anandamide): their affinities for cannabinoid receptors and pathways of inactivation. *Molecular Pharmacology* 1998; **54**:180-188.

EL-Gohary M, Eid M. Effect of cannabinoid ingestion (in the form of bhang) on the immune system of high school and university students. *Human & Experimental Toxicology* 2004; **23**:149-156.

Ellert-Miklaszewska A, Kaminska B, Konarska L. Cannabinoids down-regulate PI3K/AKT and ERK signalling pathways and activate proapoptotic function of Bad protein. *Cellular Signalling* 2005; **17**:25-37.

Elsohly MA, Slade D. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sciences* 2005; **78**:539-548.

English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb M. New insights into the control of MAP kinase pathways. *Experimental Cell Research* 1999; **253**:255-270.

Facchinetti F, Del Giudice E, Furegato S, Passarotto M, Leon A. Cannabinoids ablate release of TNFα in rat microglial cells stimulated with lypopolysaccharide. *Glia* 2003; **41**:161-168.

Faubert Kaplan B, Kaminski N. Cannabinoids inhibit the activation of ERK MAPK in PMA/Io-stimulated mouse splenocytes. *International Immunopharmacology* 2003; **3**:1503-1510.

Faubert B, Kaminski N. AP-1 activity is negatively regulated by cannabinol through inhibition of its protein components, c-fos and c-jun. *Journal of Leukocyte Biology* 2000; **67**:259-266.

Felder C, Veluz J, Williams H, Briley E, Matsuda L. Cannabinoid agonists stimulate both receptor and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Molecular Pharmacology* 1992; **42**:838-845.

Felder C, Joyce K, Briley E, Glass M, Mackie K, Fahey K, Cullinan G, Hunden D, Johnson D, Chaney M, Koppel G, Brownstein M. LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. *Journal of Pharmacology and Experimental Therapeutics* 1998; **284**:291-297.

Ferguson S. Evolving concepts in G-protein coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacological Reviews* 2001; **53**:1-24.

Fernando S, Pertwee R. Evidence that methyl arachidonyl fluorophosphonate is an irreversible cannabinoid receptor antagonist. *British Journal of Pharmacology* 1997; **121**:1716-1720.

Fezza F, Bisogno T, Minassi A, Appendino G, Mechoulam R, Di Marzo V. Noladin ether, a putative novel endocannabinoid: inactivation mechanisms and a sensitive method for its quantification in rat tissues. *FEBS Letters* 2002; **513**:294-298.

Filppula S, Yaddanapudi S, Mercier R, Xu W, Pavlopoulos S, Makriyannis A. Purification and mass spectroscopic analysis of human CB2 cannabinoid receptor expressed in the baculovirus system. *Journal of Peptide Research* 2004; **64**:225-236.

Fimiani C, Mattocks D, Cavani F, Salzet M, Deutsch G, Pryor S, Bilfinger T, Stefano G. Morphine and anandamide stimulate intracellular calcium transients in human arterial endothelial cells: coupling to nitric oxide release. *Cellular Signalling* 1999; **11**:189-193.

Flygare J, Gustafsson K, Kimby E, Christensson B, Sander B. Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma. *FEBS Letters* 2005; **579**:6885-6889.

Foster F, Traer C, Abraham S, Fry M. The phosphoinositide (PI) 3-kinase family. *Journal of Cell Science* 2003; **116**:3037-3040.

Franklin A, Parmentier-Batteur S, Walter L, Greenberg D, Stella N. Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *Journal of Neuroscience* 2003; **23**:7767-7775.

Franklin A, Stella N. Arachidonylcyclopropylamide increases microglial cell migration through cannabinoid CB2 and abnormal-cannabidiol-sensitive receptors. *European Journal of Pharmacology* 2003; **474**:195-198.

Fukata M, Nakagawa M, Kaibuchi K. Roles of Rho-family GTPases in cell polarisation and directional migration. *Current Opinion in Cell Biology* 2003; **15**:590-597.

Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *European Journal of Biochemistry* 1995; **232**:54-61.

Galve-Roperh I, Rueda D, Gomez P, Velasco G, Guzman M. Mechanism of extracellular signal-regulated kinase activation by the CB(1) cannabinoid receptor. *Molecular Pharmacology* 2002; **62**:1385-1392.

Galve-Roperh I, Sanchez C, Cortes M, Del Pulgar T, Izquierdo M, Guzman M. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nature Medicine* 2000; **6**:313-319.

Gao T, Furnari F, Newton AC. PHLPP: A phosphatase that directly dephosphorylates AKT, promotes apoptosis, and suppresses tumor growth. *Molecular Cell* 2005; **18**:13-24.

Gaoni Y , Mechoulam R. Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *Journal of the American Chemical Society* 1964; **86:** 1646-1647.

Garcia D, Brown S, Hille B, Mackie K. Protein kinase C disrupts cannabinoid actions by phosphorylation of the CB1 cannabinoid receptor. *Journal of Neuroscience* 1998; **18**:2834-2841.

Gardner B, Zu L, Sharma S, Liu Q, Makriyannis A, Tashkin D, Dubinett S. Autocrine and paracrine regulation of lymphocyte CB2 receptor expression by TGFβ. *Biochemical and Biophysical Research Communications* 2002; **290**:91-96.

Gerard C, Mollereau C, Vassart G, Parmentier M. Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochemical Journal* 1991; **279**:129-134.

Germain R. T-cell development and the CD4-CD8 lineage decision. *Nature Reviews in Immunology* 2002; **2**:309-322.

Gertsch J, Schoop R, Kuenzle U, Suter A. Echinacea alkylamides modulate TNFα gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS Letters* 2004; **577**:563-569.

Ghosh S, Preet A, Groopman J, Ganju R. Cannabinoid receptor CB(2) modulates the CXCL12/CXCR4-mediated chemotaxis of T lymphocytes. *Molecular Immunology* 2006; **43**: 2169-2179.

Gifford A, Bruneus M, Gatley S, Lan R, Makriyannis A, Volkow N. Large receptor reserve for cannabinoid actions in the central nervous system. *Journal of Pharmacology and Experimental Therapeutics* 1999; **288**:478-483.

Giuffrida A, Beltramo M, Piomelli D. Mechanisms of endocannabinoid inactivation: biochemistry and pharmacology. *Journal of Pharmacology and Experimental Therapeutics* 2001; **298**:7-14.

Glaser S, Abumrad N, Fatade F, Kaczocha M, Studholme K, Deutsch D. Evidence against the presence of an anandamide transporter. *Proceedings of the National Academy of Sciences, U.S.A* 2003; **100**:4269-4274.

Glass M, Felder C. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *Journal of Neuroscience* 1997; **17**:5327-5333.

Glass M, Northup J. Agonist selective regulation of G-proteins by cannabinoid CB1 and CB2 receptors. *Molecular Pharmacology* 1999; **56**:1362-1369.

Godfrey D, MacDonald H, Kronenberg M, Smyth M, Van K. NKT cells: what's in a name? *Nature Reviews in Immunology* 2004; **4**:231-237.

Gokoh M, Kishimoto S, Oka S, Metani Y, Sugiura T. 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, enhances the adhesion of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes. *FEBS Letters* 2005a; **579**:6473-6478.

Gokoh M, Kishimoto S, Oka S, Mori M, Waku K, Ishima Y, Sugiura T. 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces rapid actin polymerization in HL-60 cells differentiated into macrophage-like cells. *Biochemical Journal* 2005b; **386**:583-589.

Golech S, McCarron R, Chen Y, Bembry J, Lenz F, Mechoulam R, Shohami E, Spatz M. Human brain endothelium: coexpression and function of vanilloid and endocannabinoid receptors. *Molecular Brain Research* 2004; **132**:87-92.

Gomez P, De Ceballos M, Guzman M, Velasco G. Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. *Journal of Biological Chemistry* 2002; **277**:36527-36533.

Gomez P, Velasco G, Guzman M. The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/AKT. *Biochemical Journal* 2000; **347**:369-373.

Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin W. Endocannabinoid 2-arachidonylglycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. *Molecular Pharmacology* 2000; **57**: 1045-1050

Goparaju S, Ueda N, Taniguchi K, Yamamoto S. Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochemical Pharmacology* 1999; **57**:417-423.

Goparaju S, Ueda N, Yamaguchi H, Yamamoto S. Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. *FEBS Letters* 1998; **422**:69-73.

Goutopoulos A, Makriyannis A. From cannabis to cannabinergics: new therapeutic opportunities. *Pharmacology & Therapeutics* 2002; **95**:103-117.

Grimaldi C, Pisanti S, Laezza C, Malfitano A, Santoro A, Vitale M, Caruso M, Notarnicola M, Iacuzzo I, Portella G, Di Marzo V, Bifulco M. Anandamide inhibits adhesion and migration of breast cancer cells. *Experimental Cell Research* 2006; **312**:363-373.

Gschwendt M, Muller H, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F. Rottlerin, a novel protein kinase inhibitor. *Biochemical and Biophysical Research Communications* 1994; **199**:93-98.

Guzman M, Sanchez C. Effects of cannabinoids on energy metabolism. *Life Sciences* 1999; **65**:657-664.

Guzman M, Sanchez C, Galve-Roperh I. Cannabinoids and cell fate. *Pharmacology & Therapeutics* 2002; **95**:175-184.

Hajos N, Katona I, Naiem S, Mackie K, Ledent C, Mody I, Freund T. Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *European Journal of Neuroscience* 2000; **12**:3239-3249.

Hajos N, Kathuria S, Dinh T, Piomelli D, Freund T. Endocannabinoid transport tightly controls 2-arachidonoyl glycerol actions in the hippocampus: effects of low temperature and the transport inhibitor AM404. *European Journal of Neuroscience* 2004; **19**:2991-2996.

Hansen J, Sheikh S. Functional consequences of 7TM receptor dimerization. *European Journal of Pharmacoligcal Sciences* 2004; **23**:301-317.

Hanus L, Abu-Lafi S, Fride E, Breuer A, Vogel Z, Shalev D, Kustanovich I, Mechaoulam R. 2-Arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proceedings of the National Academy of Sciences, USA* 2001; **98**:3662-3665.

Hart S, Fischer O, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. *Cancer Research* 2004; **64**:1943-1950.

Hay D, Poyner D, Sexton P. GPCR modulation by RAMPs. *Pharmacology & Therapeutics* 2006; **109**:173-197.

Hennecke J, Wiley D. T cell receptor-MHC interactions up close. *Cell* 2001; **104**:1-4.

Hermann A, Kaczocha M, Deutsch D. 2-Arachidonoylglycerol (2-AG) membrane transport: history and outlook. *AAPS Journal* 2006; **8**:E409-E412.

Herrera B, Carracedo A, Ez-Zaera M, Guzman M, Velasco G. p38 MAPK is involved in CB2 receptor-induced apoptosis of human leukaemia cells. *FEBS Letters* 2005; **579**:5084-5088.

Herring A, Faubert Kaplan B, Kaminski N. Modulation of CREB and NFκB signal transduction by cannabinol in activated thymocytes. *Cellular Signalling* 2001; **13**:241-250.

Herring A, Kaminski N. Cannabinol-mediated inhibition of nuclear factor-κB, cAMP response element-binding protein, and interleukin-2 secretion by activated thymocytes. *Journal of Pharmacology and Experimental Therapeutics* 1999; **291**:1156-1163.

Hilairet S, Bouaboula M, Carriere D, Le Fur G, Casellas P. Hypersensitization of the orexin 1 receptor by the CB1 receptor: evidence for cross-talk blocked by the specific CB1 antagonist SR141716. *Journal of Biological Chemistry* 2003; **278**:23731-23737.

Hillard C, Jarrahian A. Cellular accumulation of anandamide: consensus and controversy. *British Journal of Pharmacology* 2003; **140**:802-808.

Hillard C, Manna S, Greenberg M, Di Camelli R, Ross R, Stevenson L, Murphy V, Pertwee R, Campbell W. Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *Journal of Pharmacology and Experimental Therapeutics* 1999; **289**:1427-1433.

Hinz B, Ramer R, Eichele K, Weinzierl U, Brune K. R(+)-methanandamide-induced cyclooxygenase-2 expression in H4 human neuroglioma cells: possible involvement of membrane lipid rafts. *Biochemical and Biophysical Research Communications* 2004; **324**:621-626.

Howe A. Regulation of actin-based cell migration by cAMP/PKA. *Biochimica et Biophysica Acta Molecular Cell Research* 2004; **1692**:159-174.

Howlett A, Barth F, Bonner T, Cabral G, Casellas P, Devane W, Felder C, Herkenham M, Mackie K, Martin B, Mechoulam R, Pertwee R. International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacological Reviews* 2002; **54**:161-202.

Howlett A, Mukhopadhyay S. Cellular signal transduction by anandamide and 2-arachidonoylglycerol. *Chemistry and Physics of Lipids* 2000; **108**:53-70.

Hsieh C, Brown S, Derleth C, Mackie K. Internalization and recycling of the CB1 cannabinoid receptor. *Journal of Neurochemistry* 1999; **73**:493-501.

Huang C, Jacobson K, Schaller M. MAP kinases and cell migration. *Journal of Cell Science* 2004; **117**:4619-4628.

Huang S, Bisogno T, Trevisani M, Al-Hayani A, De Petrocellis L, Fezza F, Tognetto M, Petros T, Krey J, Chu C, Miller J, Davies S, Geppetti P, Walker M, Di Marzo V. An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. *Proceedings of the National Academy of Sciences, USA* 2002; **99**:8400-8405.

Hwang S, Oh U. Hot channels in airways: pharmacology of the vanilloid receptor. *Current Opinion in Pharmacology* 2002; **2**:235-242.

Ihenetu K, Molleman A, Parsons M, Whelan C. Pharmacological characterisation of cannabinoid receptors inhibiting interleukin 2 release from human peripheral blood mononuclear cells. *European Journal of Pharmacology* 2003a; **464**:207-215.

Ihenetu K, Molleman A, Parsons M, Whelan C. Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. *European Journal of Pharmacology* 2003b; **458**:207-215.

Infantino S, Moepps B, Thelen M. Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells. *Journal of Immunology* 2006; **176**:2197-2207.

Iversen L. Cannabis and the brain. *Brain* 2003; **126**:1252-1270.

Izzo A, Fezza F, Capasso R, Bisogno T, Pinto L, Iuvone T, Esposito G, Mascolo N, Di Marzo V, Capasso F. Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *British Journal of Pharmacology* 2001; **134**:563-570.

Jan T, Rao G, Kaminski N. Cannabinol enhancement of interleukin-2 (IL-2) expression by T cells is associated with an increase in IL-2 distal nuclear factor of activated T cell activity. *Molecular Pharmacology* 2002; **61**:446-454.

Jan T, Kaminski N. Role of mitogen-activated protein kinases in the differential regulation of interleukin-2 by cannabinol. *Journal of Leukocyte Biology* 2001; **69**:841-849.

Janeway C, Travers P, Walport M, Capra J. *Immunobiology: the immune system in health and disease*, Fourth Edition, Garland Scientific Publishing, 1999.

Jarrahian A, Watts V, Barker E. D2 dopamine receptors modulate Gα-subunit coupling of the CB1 cannabinoid receptor. *Journal of Pharmacology and Experimental Therapeutics* 2004; **308**:880-886.

Jbilo O, Derocq J, Segui M, Le Fur G, Casellas P. Stimulation of peripheral cannabinoid receptor CB2 induces MCP-1 and IL-8 gene expression in human promyelocytic cell line HL60. *FEBS Letters* 1999; **448**:273-277.

Jeon Y, Yang K, Pulaski J, Kaminski N. Attenuation of inducible nitric oxide synthase gene expression by Δ^9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor-κB/Rel activation. *Molecular Pharmacology* 1996; **50**:334-341.

Jin W, Brown S, Roche J, Hsieh C, Celver J, Kavoor A, Chavkin C, Mackie K. Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization. *Journal of Neuroscience* 1999; **19**:3773-3780.

Johnston B, Butcher E. Chemokines in rapid leukocyte adhesion triggering and migration. *Seminars in Immunology* 2002; **14**:83-92.

Jorda M, Rayman N, Tas M, Verbakel S, Battista N, Van Lom K, Lowenberg B, Maccarrone M, Delwel R. The peripheral cannabinoid receptor Cb2, frequently expressed on AML blasts, either induces a neutrophilic differentiation block or confers abnormal migration properties in a ligand-dependent manner. *Blood* 2004.

Jorda M, Verbakel S, Valk P, Vankan-Berkhout Y, Maccarrone M, Finazzi-Agro A, Lowenberg B, Delwel R. Hematopoietic cells expressing the peripheral cannabinoid receptor migrate in response to the endocannabinoid 2-arachidonoylglycerol. *Blood* 2002; **99**:2786-2793.

Jorda M, Lowenberg B, Delwel R. The peripheral cannabinoid receptor Cb2, a novel oncoprotein, induces a reversible block in neutrophilic differentiation. *Blood* 2003; **101**:1336-1343.

Joseph J, Niggemann B, Zaenker K, Entschladen F. Anandamide is an endogenous inhibitor for the migration of tumor cells and T lymphocytes. *Cancer Immunology & Immunotherapy* 2004; **53**:723-728.

Juttler E, Potrovita I, Tarabin V, Prinz S, Dong-Si T, Fink G, Schwaniger M. The cannabinoid dexanabinol is an inhibitor of the nuclear factor-κB (NFκB). *Neuropharmacology* 2004; **47**:580-592.

Kaminski N. Regulation of the cAMP cascade, gene expression and immune function by cannabinoid receptors. *Journal of Neuroimmunology* 1998; **83**:124-132.

Kaplan B, Ouyang Y, Rockwell C, Rao GK, Kaminski N. 2-Arachidonoyl-glycerol suppresses interferon-γ production in phorbol ester/ionomycin-activated mouse splenocytes independent of CB1 or CB2. *Journal of Leukocyte Biology* 2005a; **77**:966-974.

Kaplan B, Ouyang Y, Herring A, Su Yea S, Razdan R, Kaminski N. Inhibition of leukocyte function and interleukin-2 gene expression by 2-methylarachidonyl-(2'-fluoroethyl)amide, a stable congener of the endogenous cannabinoid receptor ligand anandamide. *Toxicology and Applied Pharmacology* 2005b; **205**:107-115.

Kaplan B, Rockwell C, Kaminski N. Evidence for cannabinoid receptor-dependent and independent mechanisms of action in leukocytes. *Journal of Pharmacology and Experimental Therapeutics* 2003; **306**:1077-1085.

Karanian D, Brown Q, Makriyannis A, Bahr B. Blocking cannabinoid activation of FAK and ERK1/2 compromises synaptic integrity in hippocampus. *European Journal of Pharmacology* 2005; **508**:47-56.

Karlsson M, Contreras JA, Hellman U, Tornqvist H, Holm C. cDNA Cloning, Tissue Distribution, and Identification of the Catalytic Triad of Monoglyceride Lipase. Evolutionary relationship to esterases, lysophopholipases and haloperoxidases. *Journal of Biological Chemistry* 1997; **272**:27218-27223.

Karlsson M, Pahlsson C, Fowler C. Reversible, temperature-dependent, and AM404-inhibitable adsorption of anandamide to cell culture wells as a confounding factor in release experiments. *European Journal of Pharmaceutical Sciences* 2004; **22**:181-189.

Karlsson M, Reue K, Xia Y, Lusis A, Langin D, Tornqvist H, Holm C. Exon-intron organization and chromosomal localization of the mouse monoglyceride lipase gene. *Gene* 2001; **272**:11-18.

Katona I, Rancz E, Acsady L, Ledent C, Mackie K, Hajos N, Freund T. Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *Journal of Neuroscience* 2001; **21**:9506-9518.

Kearn C, Blake-Palmer K, Daniel E, Mackie K, Glass M. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors enhances heterodimer formation: a mechanims for receptor cross-talk? *Molecular Pharmacology* 2005; **67**:1697-1704.

Keren O, Sarne Y. Multiple mechanisms of CB1 cannabinoid receptors regulation. *Brain Research* 2003; **980**:197-205.

Kirfel G, Rigort A, Borm B, Herzog V. Cell migration: mechanisms of rear detachment and the formation of migration tracks. *European Journal of Cell Biology* 2004; **83**:717-724.

Kishimoto S, Gokoh M, Oka S, Maramatsu M, Kajiwara T, Waku K, Sugiura T. 2-arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB2 receptor-dependent mechanism. *Journal of Biological Chemistry* 2003; **278**:24469-24475.

Klein T, Friedman H, Specter S. Marijuana, immunity and infection. *Journal of Neuroimmunology* 1998a; **83**:102-115.

Klein T, Newton C, Larsen K, Lu L, Perkins I, Nong L, Friedman M. The cannabinoid system and immune modulation. *Journal of Leukocyte Biology* 2003; **74**:486-496.

Klein T, Newton C, Nakachi N, Friedman H. Δ^9-Tetrahydrocannabinol treatment suppresses immunity and early IFN-γ, IL-12, and IL-12 receptor β2 responses to *Legionella pneumophila* infection. *Journal of Immunology* 2000; **164**:6461-6466.

Klein T. Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nature Reviews in Immunology* 2005; **5**:400-411.

Klein T, Newton C, Friedman H. Cannabinoid receptors and immunity. *Immunology Today* 1998b; **19**:373-381.

Klein T, Newton C, Larsen K, Chou J, Perkins I, Lu L, Nong L, Friedman H. Cannabinoid receptors and T helper cells. *Journal of Neuroimmunology* 2004; **147**:91-94.

Klemke R, Cai S, Giannini A, Gallagher P, Lanerolle P, Cheresh D. Regulation of cell motility by mitogen-activated protein linase. *Journal of Cell Biology* 1997; **137**:481-492.

Knight Z, Chiang G, Alaimo P, Kenski D, Ho C, Coan K, Abraham R, Shokat K. Isoform-specific phosphoinositide 3-kinase inhibitors from an arylmorpholine scaffold. *Bioorganic & Medicinal Chemistry* 2004; **12**:4749-4759.

Kobayashi Y, Arai S, Waku K, Sugiura T. Activation by 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, of p42/44 mitogen-activated protein kinase in HL-60 cells. *Journal of Biochemistry* 2001; **129**:665-669.

Koh W, Crawford R, Kaminski N. Inhibition of protein kinase A and cyclic AMP response element (CRE)-specific transcription factor binding by Δ^9-tetrahydrocannabinol (Δ^9-THC) - A putative mechanism of cannabinoid-induced immune modulation. *Biochemical Pharmacology* 1997; **53**:1477-1484.

Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochemical Journal* 2000; **351**:289-305.

Kopperud R, Krakstad C, Selheim F, Doskeland S. cAMP effector mechanisms. Novel twists for an 'old' signaling system. *FEBS Letters* 2003; **546**:121-126.

Kozak K, Crews B, Morrow J, Wang L, Ma Y, Weinander R, Jakobsson P, Marnett L. Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *Journal of Biological Chemistry* 2002a; **277**:44877-44885.

Kozak K, Crews B, Ray J, Tai H, Morrow J, Marnett L. Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamides *in vitro* and *in vivo*. *Journal of Biological Chemistry* 2001; **276**:36993-36998.

Kozak K, Gupta R, Moody J, Ji C, Boeglin W, DuBois R, Brash A, Marnett L. 15-Lipoxygenase metabolism of 2-arachidonylglycerol. Generation of a peroxisome proliferator-activated receptor-α agonist. *Journal of Biological Chemistry* 2002b; **277**:23278-23286.

Kozak K, Marnett L. Oxidative metabolism of endocannabinoids. *Prostaglandins Leukotrienes and Essential Fatty Acids* 2002; **66**:211-220.

Kristiansen K. Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacology & Therapeutics* 2004; **103**:21-80.

Kroczek R, Mages H, Hutloff A. Emerging paradigms of T-cell co-stimulation. *Current Opinion in Immunology* 2004; **16**:321-327.

Krummel M, Sullivan T, Allison J, Lanier L. Superantigen responses and co-stimulation: CD28 and CTLA-4 have opposing effects on T cell expansion *in vitro* and *in vivo*. *International Immunology* 1996; **8**:519-523.

Kurihara R, Tohyama Y, Matsusaka S, Naruse H, Kinoshita E, Tsujiota T, Katsumata Y, Yamamura H. Effects of peripheral cannabionoid receptor ligands on motility and polarization in neutrophil-like HL60 cells and human neutrophils. *Journal of Biological Chemistry* 2006; **281**: 12908-12918.

Lanzafame A, Guida E, Christopoulos A. Effects of anandamide on the binding and signaling properties of M1 muscarinic acetylcholine receptors. *Biochemical Pharmacology* 2004; **68**:2207-2219.

Lanzavecchia A, Sallusto F. Understanding the generation and function of memory T cell subsets. *Current Opinion in Immunology* 2005; **17**:326-332.

Larsson C. Protein kinase C and the regulation of the actin cytoskeleton. *Cellular Signalling* 2006; **18**:276-284.

Lauckner J, Hille B, Mackie K. The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. *Proceedings of the National Academy of Sciences, U.S.A* 2005; **102**:19144-19149.

Laudanna C, Alon R. Right on the spot. Chemokine triggering of integrin-mediated arrest of rolling leukocytes. *Thrombosis and Haemostasis* 2006; **95**:5-11.

Lee S, Newton C, Widen R, Friedman H, Klein T. Differential expression of cannabinoid CB(2) receptor mRNA in mouse immune cell subpopulations and

following B cell stimulation. *European Journal of Pharmacology* 2001; **423**:235-241.

Leterrier C, Bonnard D, Carrel D, Rossier J, Lenkei Z. Constitutive endocytic cycle of the CB1 cannabinoid receptor. *Journal of Biological Chemistry* 2004; **279**:36013-36021.

Ligresti A, Bisogno T, Matias I, De Petrocellis L, Cascio M, Cosenza V, D'Argenio G, Scalione G, Bifulco M, Sorrentini L, Di Marzo V. Possible endocannabinoid control of colorectal cancer growth. *Gastroenterology* 2003; **125**:677-687.

Liu J, Gao B, Mirshahi F, Sanyal A, Khanolkar A, Makriyannis A, Kunos G. Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochemical Journal* 2000; **346**:835-840.

Liu J, Li H, Burstein S, Zurier R, Chen J. Activation and binding of peroxisome proliferator-activated receptor-γ by synthetic cannabinoid ajulemic acid. *Molecular Pharmacology* 2003; **63**:983-992.

Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D. The nuclear receptor peroxisome proliferator-activated receptor-α mediates the anti-inflammatory actions of palmitoylethanolamide. *Molecular Pharmacology* 2005a; **67**:15-19.

Lo Verme J, La Rana G, Russo R, Calignano A, Piomelli D. The search for the palmitoylethanolamide receptor. *Life Sciences* 2005b; **77**:1685-1698.

Lombard C, Nagarkatti M, Nagarkatti P. Targeting cannabinoid receptors to treat leukemia: role of cross-talk between extrinsic and intrinsic pathways in Δ^9-tetrahydrocannabinol (THC)-induced apoptosis of Jurkat cells. *Leukemia Research* 2005; **29**:915-922.

Los M, Schenk H, Hexel K, Baeuerle P, Droge W, Schulze-Osthoff K. IL-2 gene expression and NFκB activation through CD28 requires reactive oxygen production by 5-lipoxygenase. *EMBO Journal* 1995; **14**: 3731-3740.

Lozovaya N, Yatsenko N, Beketov A, Tsintsadze T, Burnashev N. Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. *Journal of Neuroscience* 2005; **25**:7499-7506.

Luo Y, Patel M, Wiederhold M, Ou D. Effects of cannabinoids and cocaine on the mitogen-induced transformations of lymphocytes of human and mouse origins. *International Journal of Immunopharmacology* 1992; **14**:49-56.

Luster A, Alon R, von Andrian U. Immune cell migration in inflammation: present and future therapeutic targets. *Nature Immunology* 2005; **6**:1182-1190.

Lynn A, Herkenham M. Localization of cannabinoid receptors and nonsaturable high-density cannabinoid binding sites in peripheral tissues of the rat: implications for receptor-mediated immune modulation by cannabinoids. *Journal of Pharmacology and Experimental Therapeutics* 1994; **268**:1612-1623.

Maccarrone M, De Petrocellis L, Bari M, Fezza F, Salvati S, Di Marzo V, Finazzi-Agro A. Lipopolysaccharide downregulates fatty acid amide hydrolase expression and increases anandamide levels in human peripheral lymphocytes. *Archives of Biochemistry and Biophysics* 2001; **393**:321-328.

Maccarrone M, Lorenzon T, Bari M, Melino G, Finazzi-Agro A. Anandamide induces apoptosis in human cells via vanilloid receptors - Evidence for a protective role of cannabinoid receptors. *Journal of Biological Chemistry* 2000; **275**:31938-31945.

Maccarrone M, Bari M, Di Rienzo M, Finazzi-Agro A, Rossi A. Progesterone activates fatty acid amide hydrolase (FAAH) promoter in human T lymphocytes through the transcription factor Ikaros: evidence for a synergistic effect of leptin. *Journal of Biological Chemistry* 2003a; **278**:32726-32732.

Maccarrone M, Di Rienzo M, Battista N, Gasperi V, Guerrieri P, Rossi A, Finazzi-Agro A. The endocannabinoid system in human keratinocytes: evidence that ananadamide inhibits epidermal differentiation through CB1 receptor-dependent inhibition of protein kinase C, activating protein-1 and transglutaminase. *Journal of Biological Chemistry* 2003b; **278**:33896-33903.

Maccarrone M, Di Rienzo M, Finazzi-Agro A, Rossi A. Leptin activates the anandamide hydrolase promoter in human T lymphocytes through STAT3. *Journal of Biological Chemistry* 2003c; **278**:13318-13324.

MacDonald T, Monteleone G. Immunity, inflammation, and allergy in the gut. *Science* 2005; **307**:1920-1925.

Mackie K. Cannabinoid receptor homo- and heterodimerization. *Life Sciences* 2005; **77**:1667-1673.

Maestroni G. The endogenous cannabinoid 2-arachidonoyl glycerol as *in vivo* chemoattractant for dendritic cells and adjuvant for Th1 response to a soluble protein. *FASEB Journal* 2004; **18**: 1914-1916.

Maggio R, Novi F, Scarselli M, Corsini G. The impact of G-protein-coupled receptor hetero-oligomerization on function and pharmacology. *FEBS Journal* 2005; **272**:2939-2946.

Maier U, Babich A, Nurnberg B. Roles of non-catalytic subunits in Gβγ-induced activation of class I phosphoinositide 3-kinase isoforms β and γ. *Journal of Biological Chemistry* 1999; **274**:29311-29317.

Makriyannis A, Tian X, Guo J. How lipophilic cannabinergic ligands reach their receptor sites. *Prostaglandins &Other Lipid Mediators* 2005; **77**:210-218.

Maneuf Y, Brotchie J. Paradoxical action of the cannabinoid WIN 55,212-2 in stimulated and basal cyclic AMP accumulation in rat globus pallidus slices. *British Journal of Pharmacology* 1997; **120**:1397-1398.

Marchand J, Bord A, Penarier G, Laure F, Carayon P, Casellas P. Quantitative method to determine mRNA levels by reverse transcriptase polymerase chain reaction from leukocyte subsets purified by fluorescence-activated cell sorting: application to peripheral cannabinoid receptors. *Cytometry* 1999; **35**:227-234.

Maresz K, Carrier E, Ponomarev E, Hillard C, Dittel B. Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. *Journal of Neurochemistry* 2005; **95**:437-445.

Marinissen M, Gutkind J. G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends in Pharmacological Sciences* 2001; **22**:368-376.

Martin B, Wiley J, Beletskaya I , Sim-Selley L, Smith F, Dewey W, Cottney J, Adams J, Baker J, Hill D, Saha B, Zerkowski J, Mahadevan A, Razdan R. Pharmacological characterization of novel water-soluble cannabinoids. *Journal of Pharmacology and Experimental Therapeutics* 2006; **381**: 1231239.

Martin B, Sim-Selley L, Selley D. Signaling pathways involved in the development of cannabinoid tolerance. *Trends in Pharmacological Sciences* 2004; **25**:325-330.

Martiny-Baron G, Kazanietz M, Mischak H, Blumberg P, Kochs G, Hug H, Marme D, Schachtele C. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö6976. *Journal of Biological Chemistry* 1993; **268**:9194-9197.

Massi P, Sacerdote P, Ponti W, Fuzio D, Manfredi B, Vigano D, Rubino T, Bardoti M, Parolaro D. Immune function alterations in mice tolerant to Δ^9-tetrahydrocannabinol: functional and biochemical parameters. *Journal of Neuroimmunology* 1998; **92**:60-66.

Matias I, Pochard P, Orlando P, Salzet M, Pestel J, Di Marzo V. Presence and regulation of the endocannabinoid system in human dendritic cells. *European Journal of Biochemistry* 2002; **269**:3771-3778.

Matsuda L, Lolait S, Brownstein M, Young A, Bonner T. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990; **346**:561-564.

Matthews S, Cantrell D. The role of serine/threonine kinases in T-cell activation. *Current Opinion in Immunology* 2006; **18**:314-320.

McFarland M, Porter A, Rakhshan F, Rawat D, Gibbs R, Barker E. A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide. *Journal of Biological Chemistry* 2004; **279**:41991-41997.

McKallip R, Lombard C, Fisher M, Martin B, Ryu S, Grant S, Nagarkatti P, Nagarkatti M. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood* 2002a; **100**:627-634.

McKallip R, Lombard C, Martin B, Nagarkatti M, Nagarkatti P. Δ^9-Tetrahydrocannabinol-induced apoptosis in the thymus and spleen as a mechanism of immunosuppression *in vitro* and *in vivo*. *Journal of Pharmacology and Experimental Therapeutics* 2002b; **302**:451-465.

Mechoulam R, Ben Shabat S, Hanus L, Ligumsky M, Kaminski N, Schatz A, Gopher A, Almog S, Martin B, Compton D, Pertwee R, Griffin G, Bayewitch M, Barg J, Vogel Z. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemal Pharmacology* 1995; **50**:83-90.

Melck D, Rueda D, Galve-Roperh I, De Petrocellis L, Guzman M, Di Marzo V. Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-proliferative effects of anandamide in human breast cancer cells. *FEBS Letters* 1999; **463**:235-240.

Mestas J, Hughes C. Of mice and not men: differences between mouse and human immunology. *Journal of Immunology* 2004; **172**:2731-2738.

Ming Mo F, Offertaler L, Kunos G. Atypical cannabinoid stimulates endothelial cell migration via a Gi/Go-coupled receptor distinct from CB1, CB2 or EDG-1. *European Journal of Pharmacology* 2004; **489**: 21-27

Moelling K, Schad K, Bosse M, Zimmermann S, Schweneker M. Regulation of Raf-Akt cross-talk. *Journal of Biological Chemistry* 2002; **277**:31099-31106.

Molina-Holgado E, Vela J, Arevalo-Martin A, Almazan G, Molina-Holgado F, Borell J, Guaza C. Cannabinoids promote oligodendrocytepProgenitor survival: involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/AKT signaling. *Journal of Neuroscience* 2002; **22**:9742-9753.

Molina-Holgado F, Pinteaux E, Heenan L, Moore J, Rothwell N, Gibson R. Neuroprotective effects of the synthetic cannabinoid HU-210 in primary cortical neurons are mediated by phosphatidylinositol 3-kinase/AKT signaling. *Molecular and Cellular Neuroscience* 2005; **28**:189-194.

Mombouli J, Schaeffer G, Holzmann S, Kostner G, Graier W. Anandamide-induced mobilization of cytosolic Ca^{2+} in endothelial cells. *British Journal of Pharmacology* 1999; **126**:1593-1600.

Moody J, Kozak K, Ji C, Marnett L. Selective oxygenation of the endocannabinoid 2-arachidonylglycerol by leukocyte-type 12-lipoxygenase. *Biochemistry* 2001; **40**:861-866.

Moore S, Nomikos G, Dickason-Chesterfield A, Schober D, Schaus J, Ying B, Xu Y, Phebus L, Simmons R, Li D, Iyengar S, Felder C. Identification of a high-affinity binding site involved in the transport of endocannabinoids. *Proceedings of the National Academy of Sciences, USA* 2005; **102:** 17852-17857.

Movahed P, Jonsson B, Birnir B, Wingstrand J, Jorgesen T, Ermund A, Sterner O, Zygmunt P, Hogestatt E. Endogenous unsaturated C18 *N*-acylethanolamines are vanilloid receptor (TRPV1) agonists. *Journal of Biological Chemistry* 2005; **280**:38496-38504.

Mukherjee S, Adams M, Whiteaker K, Daza A, Kage K, Cassar S, Meyer M, Yao B. Species comparison and pharmacological characterization of rat and human CB2 cannabinoid receptors. *European Journal of Pharmacology* 2004; **505**:1-9.

Mukhopadhyay S, Howlett A. CB1 receptor-G protein association. Subtype selectivity is determined by distinct intracellular domains. *European Journal of Biochemistry* 2001; **268**:499-505.

Mukhopadhyay S, Howlett A. Chemically distinct ligands promote differential CB1 cannabinoid receptor-Gi protein interactions. *Molecular Pharmacology* 2005; **67**:2016-2024.

Mukhopadhyay S, McIntosh H, Houston D, Howlett A. The CB1 cannabinoid receptor juxtamembrane C-terminal peptide confers activation to specific G proteins in brain. *Molecular Pharmacology* 2000; **57**:162-170.

Mukhopadhyay S, Shim J, Assi A, Norford D, Howlett A. CB1 cannabinoid receptor-G protein association: a possible mechanism for differential signaling. *Chemistry and Physics of Lipids* 2002; **121**:91-109.

Munro S, Thomas K, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993; **365**:61-65.

Muraille E, de Smedt T, Thielemans K, Urbain J, Moser M, Leo O. Activation of murine T cells by bacterial superantigens requires B7-mediated costimulation. *Cellular Immunology* 1995; **162**:315-320.

Murphy J, Kendall D. Integrity of extracellular loop 1 of the human cannabinoid receptor 1 is critical for high-affinity binding of the ligand CP 55,940 but not SR141716A. *Biochemical Pharmacology* 2003; **65**:1623-1631.

Nakano Y, Pross S, Friedman H. Contrasting effect of Δ^9-tetrahydrocannabinol on IL-2 activity in spleen and lymph node cells of mice of different ages. *Life Sciences* 1993; **52**:41-51.

Nelson B. IL-2, regulatory T cells, and tolerance. *Journal of Immunology* 2004; **172**:3983-3988.

Nie J, Lewis D. The proximal and distal C-terminal tail domains of the CB1 cannabinoid receptor mediate G protein coupling. *Neuroscience* 2001; **107**:161-167.

Niehaus J, Wallis K, Liu Y, Bhartur S, Elphick M, Lewis D. CRIP1a and CRIP1b: novel CB1 cannabinoid receptor interacting proteins. ICRS 14th Annual Symposium on the Cannabinoids

Nirodi C, Crews B, Kozak K, Morrow J, Marnett L. The glyceryl ester of prostaglandin E2 mobilizes calcium and activates signal transduction in RAW264.7 cells. *Proceedings of the National Academy of Sciences, USA* 2004; **101**:1840-1845.

Noe S, Newton C, Widen R, Friedman H, Klein T. Anti-CD40, anti-CD3, and IL-2 stimulation induce contrasting changes in CB1 mRNA expression in mouse splenocytes. *Journal of Neuroimmunology* 2000; **110**:161-167.

Nong L, Newton C, Cheng Q, Friedman H, Roth M, Klein T. Altered cannabinoid receptor mRNA expression in peripheral blood mononuclear cells from marijuana smokers. *Journal of Neuroimmunology* 2002; **127**:169-176.

O'Sullivan S, Tarling E, Bennett A, Kendall D, Randall M. Novel time-dependent vascular actions of Δ^9-tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor-γ. *Biochemical and Biophysical Research Communications.* 2005; **337**:824-831.

Oka S, Ikeda S, Kishimoto S, Gokoh M, Yanagimoto S, Waku K, Sugiura T. 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces the migration of EoL-1 human eosinophilic leukemia cells and human peripheral blood eosinophils. *Journal of Leukocyte Biology* 2004; **76**:1002-1009.

Oka S, Tsuchie A, Tokumura A, Muramatsu M, Suhara Y, Takayama H, Waku K, Sugiura T. Ether-linked analogue of 2-arachidonoylglycerol (noladin ether) was not detected in the brains of various mammalian species. *Journal of Neurochemistry* 2003; **85**:1374-1381.

Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nature Reviews Immunology* 2003; **3**:317-330.

Onaivi E, Chaudhuri G, Abaci A, Parker M, Manier D, Martin P, Hubbard J. Expression of cannabinoid receptors and their gene transcripts in human blood cells. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 1999; **23**:1063-1077.

Ortega-Gutierrez S, Hawkins E, Viso A, Lopez-Rodriguez M, Cravatt B. Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. *Biochemistry* 2004; **43**:8184-8190.

Ouyang Y, Hwang S, Han S, Kaminski N. Suppression of interleukin-2 by the putative endogenous cannabinoid 2-arachidonyl-glycerol is mediated through down-regulation of the nuclear factor of activated T cells. *Molecular Pharmacology* 1998; **53**:676-683.

Pablos J, Santiago B, Carriera P, Galindo M, Gomez-Reino J. Cyclooxygenase-1 and –2 are expressed by human T cells. *Clinical and Experimental Immunology* 1999; **115**: 86-90

Palmer S, Thakur G, Makriyannis A. Cannabinergic ligands. *Chemistry and Physics of Lipids* 2002; **121**:3-19.

Parker P, Murray-Rust J. PKC at a glance. *Journal of Cell Science* 2004; **117**:131-132.

Percherancier Y, Berchiche Y, Slight I, Volkmer-Engert R, Tamamura H, Fujii N, Bouvier M, Heveker N. Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *Journal of Biological Chemistry* 2005; **280**:9895-9903.

Pertwee R. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacology & Therapeutics* 1997; **74**:129-180.

Pertwee R. Cannabinoid pharmacology: the first 66 years. *British Journal of Pharmacology* 2006; **147**:S163-S171.

Pertwee R, Gibson T, Stevenson L, Ross R, Banner W, Saha B, Razdan R, Martin B. O-1057, a potent water-soluble cannabinoid receptor agonist with antinociceptive properties. *British Journal of Pharmacology* 2000; **129**:1577-1584.

Pertwee R, Ross R. Cannabinoid receptors and their ligands. *Prostaglandins Leukotrienes and Essential Fatty Acids* 2002; **66**:101-121.

Pettit D, Harrison M, Olson J, Spencer R, Cabral G. Immunohistochemical localization of the neural cannabinoid receptor in rat brain. *Journal of Neuroscience Research* 1998; **51**:391-402.

Porter A, Sauer J, Knierman M, Becker G, Berna M, Bao J, Nomikos G, Carter P, Bymaster F, Leese A, Felder C. Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 Receptor. *Journal of Pharmacology and Experimental Therapeutics* 2002; **301**:1020-1024.

Portier M, Rinaldi-Carmona M, Pecceu F, Combes T, Poinot-Chazel C, Calandra B, Barth F, Le Fur G, Casellas P. SR144528, an antagonist for the peripheral cannabinoid receptor that behaves as an inverse agonist. *Journal of Pharmacology and Experimental Therapeutics* 1999; **288**:582-589.

Pouyssegur J, Volmat V, Lenormand P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Biochemical Pharmacology* 2002; **64**:755-763.

Powles T, Poele Rt, Shamash J, Chaplin T, Propper D, Joel S, Oliver T, Liu W. Cannabis-induced cytotoxicity in leukemic cell lines: the role of the cannabinoid receptors and the MAPK pathway. *Blood* 2005; **105**:1214-1221.

Prather P, Martin N, Breivogel C, Childers S. Activation of cannabinoid receptors in rat brain by WIN 55212-2 produces coupling to multiple G protein α-subunits with different potencies. *Molecular Pharmacology* 2000; **57**:1000-1010.

Premkumar L, Qi Z, Van Buren J, Raisinghani M. Enhancement of potency and efficacy of NADA by PKC-mediated phosphorylation of vanilloid receptor. *Journal of Neurophysiology* 2004; **91**:1442-1449.

Price M, Baillie G, Thomas A, Stevenson L, Easson M, Goodwin R, McLean A, McIntosh L, Goodwin G, Walker G, Westwood P, Marrs J, Thomson F, Cowley P, Christopoulos A, Pertwee R, Ross R. Allosteric modulation of the cannabinoid CB1 receptor. *Molecular Pharmacology* 2005; **68**:1484-1495.

Prinster S, Hague C, Hall R. Heterodimerization of G protein-coupled receptors: specificity and functional significance. *Pharmacological Reviews* 2005; **57**:289-298.

Procko E, McColl S. Leukocytes on the move with phosphoinositide 3-kinase and its downstream effectors. *Bioessays* 2005; **27**:153-163.

Proft T, Fraser J. Bacterial superantigens. *Clinical and Experimental Immunology* 2003; **133**:299-306.

Pross S, Klein T, Newton C, Friedman H. Differential effects of marijuana components on proliferation of spleen, lymph node and thymus cells *in vitro*. *International Journal of Immunopharmacology* 1987; **9**:363-370.

Pross S, Klein T, Newton C, Smith J, Widen R, Friedman H. Differential suppression of T-cell subpopulations by THC (Δ^9-tetrahydrocannabinol). *International Journal of Immunopharmacology* 1990; **12**:539-544.

Pross S, Nakano Y, Widen R, McHugh S, Newton C, Klein T, Friedman H. Differing effects of Δ^9-tetrahydrocannabinol (THC) on murine spleen cell populations dependent upon stimulators. *International Journal of Immunopharmacology* 1992; **14**:1019-1027.

Puffenbarger R, Boothe A, Cabral G. Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. *Glia* 2000; **29**:58-69.

Rajagopalan G, Smart M, Marietta E, David S. Staphylococcal enterotoxin B-induced activation and concomitant resistance to cell death in CD28-deficient HLA-DQ8 transgenic mice. *International Immunology* 2002; **14**:801-812.

Rajendran L, Simons K. Lipid rafts and membrane dynamics. *Journal of Cell Science* 2005; **118**:1099-1102.

Ramer R, Brune K, Pahl A, Hinz B. R(+)-Methanandamide induces cyclooxygenase-2 expression in human neuroglioma cells via a non cannabinoid receptor-mediated mechanism. *Biochemical and Biophysical Research Communications* 2001; **286**:1144-1152.

Rayman N, Lam K, Laman J, Simons P, Lowenberg B, Sonneveld P, Delwel R. Distinct expression profiles of the peripheral cannabinoid receptor in lymphoid tissues depending on receptor activation status. *Journal of Immunology* 2004; **172**:2111-2117.

Reinhardt R, Kang S, Liang H, Locksley R. T helper cell effector fates-who, how and where? *Current Opinion in Immunology* 2006; **18**:271-277.

Revenu C, Athman R, Robine S, Louvard D. The co-workers of actin filaments: from cell structures to signals. *Nature Reviews in Molcular Cell Biology* 2004; **5**:635-646.

Rhee M, Bayewitch M, vidor-Reiss T, Levy R, Vogel Z. Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes. *Journal of Neurochemistry* 1998; **71**:1525-1534.

Ridley A, Schwartz M, Burridge K, Firtel R, Ginsberg M, Borisy G, Parsons J, Horwitz A. Cell migration: Integrating signals from front to back. *Science* 2003; **302**:1704-1709.

Rios C, Gomes I, Devi L. μ-opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. *British Journal of Pharmacology* 2006; **148**:387-395.

Rockwell C, Snider N, Thompson J, Vanden Heuvel J, Kaminski N. Interleukin-2 suppression by 2-arachidonylglycerol is mediated through peroxisome proliferator-activated receptor-γ independently of cannabinoid receptors 1 and 2. *Molecular Pharmacology* 2006; **70**:101-111.

Rockwell C, Kaminski N. A cyclooxygenase metabolite of anandamide causes inhibition of interleukin-2 secretion in murine splenocytes. *Journal of Pharmacology and Experimental Therapeutics* 2004; **311**:683-690.

Ross R. Anandamide and vanilloid TRPV1 receptors. *British Journal of Pharmacology* 2003; **140**:790-801.

Roth M, Baldwin G, Tashkin D. Effects of Δ^9-tetrahydrocannabinol on human immune function and host defense. *Chemistry and Physics of Lipids* 2002; **121**:229-239.

Rouzer C, Ghebreselasie K, Marnett L. Chemical stability of 2-arachidonylglycerol under biological conditions. *Chemistry and Physics of Lipids* 2002; **119**:69-82.

Rubino T, Forlani G, Vigano D, Zippel R, Parolaro D. Modulation of extracellular signal-regulated kinases cascade by chronic Δ^9-tetrahydrocannabinol treatment. *Molecular and Cellular Neuroscience* 2004; **25**:355-362.

Rueda D, Galve-Roperh I, Haro A, Guzman M. The CB1 cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Molecular Pharmacology* 2000; **58**:814-820.

Rueda D, Navarro B, Martinez-Serrano A, Guzman M, Galve-Roperh I. The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway. *Journal of Biological Chemistry* 2002; **277**:46645-46650.

Russo E, Burnett A, Hall B, Parker K. Agonistic properties of cannabidiol at 5-HT_{1A} receptors. *Neurochemical Research* 2005; **30**:1037-1043.

Ryberg E, Vu H, Larsson N, Groblewski T, Hjorth S, Elebring T, Sjogren S, Greasley P. Identification and characterisation of a novel splice variant of the human CB1 receptor. *FEBS Letters* 2005; **579**:259-264.

Saario S, Savinainen J, Laitinen J, Jarvinen T, Niemi R. Monoglyceride lipase-like enzymatic activity is responsible for hydrolysis of 2-arachidonoylglycerol in rat cerebellar membranes. *Biochemical Pharmacology* 2004; **67**:1381-1387.

Sacerdote P, Martucci C, Vaccani A, Boriselli F, Panerai A, Colombo A, Parolaro D, Massi P. The nonpsychoactive component of marijuana cannabidiol

modulates chemotaxis and IL-10 and IL-12 production of murine macrophages both *in vivo* and *in vitro*. *Journal of Neuroimmunology* 2005; **159**:97-105.

Sacerdote P, Massi P, Panerai A, Parolaro D. *In vivo* and *in vitro* treatment with the synthetic cannabinoid CP55,940 decreases the *in vitro* migration of macrophages in the rat: involvement of both CB1 and CB2 receptors. *Journal of Neuroimmunology* 2000; **109**:155-163.

Samelson L. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. *Annual Reviews in Immunology* 2002; **20**:371-394.

Samson M, Small-Howard A, Shimoda L, Koblan-Huberson M, Stokes A, Turner H. Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. *Journal of Immunology* 2003; **170**:4953-4962.

Sanchez C, Galve-Roperh I, Rueda D, Guzman M. Involvement of sphingomyelin hydrolysis and the mitogen-activated protein kinase cascade in the Δ^9-tetrahydrocannabinol-induced stimulation of glucose metabolism in primary astrocytes. *Molecular Pharmacology* 1998; **54**:834-843.

Sanchez C, Velasco G, Guzman M. Metabolic stimulation of mouse spleen lymphocytes by low doses of Δ^9-tetrahydrocannabinol. *Life Sciences* 1997; **60**:1709-1717.

Sanchez M, Ruiz-Llorente L, Sanchez A, Diaz-Laviada I. Activation of phosphoinositide 3-kinase/PKB pathway by CB(1) and CB(2) cannabinoid receptors expressed in prostate PC-3 cells. Involvement in Raf-1 stimulation and NGF induction. *Cellular Signaling* 2003; **15**:851-859.

Sancho R, Calzado M, Di Marzo V, Appendino G, Munoz E. Anandamide inhibits nuclear factor-κB activation through a cannabinoid receptor-independent pathway. *Molecular Pharmacology* 2003; **63**:429-438.

Sancho R, de La Vega L, Macho A, Appendino G, Di Marzo V, Munoz E. Mechanisms of HIV-1 inhibition by the lipid mediator *N*-arachidonoyldopamine. *Journal of Immunology* 2005; **175**:3990-3999.

Sancho R, Macho A, de La Vega L, Calzado M, Fiebich B, Appendino G, Munoz E. Immunosuppressive activity of endovanilloids: *N*-arachidonoyl-dopamine inhibits activation of the NF-κB, NFAT, and activator protein-1 signaling pathways. *Journal of Immunology* 2004; **172**:2341-2351.

Sarbassov D, Guertin D, Ali S, Sabatini D. Phosphorylation and regulation of AKT/PKB by the rictor-mTOR complex. *Science* 2005; **307**:1098-1101.

Sarker K, Maruyama I. Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts. *Cellular and Molecular Life Sciences* 2003; **60**:1200-1208.

Sarnataro D, Grimaldi C, Pisanti S, Gazzerro P, Laezza C, Zurzolo C, Bifulco M. Plasma membrane and lysosomal localization of CB1 cannabinoid receptor are dependent on lipid rafts and regulated by anandamide in human breast cancer cells. *FEBS Letters* 2005; **579**:6343-6349.

Schatz A, Koh W, Kaminski N. Δ^9-tetrahydrocannabinol selectively inhibits T-cell dependent humoral immune responses through direct inhibition of accessory T-cell function. *Immunopharmacology* 1993; **26**:129-137.

Schatz A, Lee M, Condie R, Pulaski J, Kaminski N. Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. *Toxicology and Applied Pharmacology* 1997; **142**:278-287.

Schlaepfer D, Mitra S, Ilic D. Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochimica et Biophysica Acta - Molecular Cell Research* 2004; **1692**:77-102.

Schmid H, Schmid P, Berdyshev E. Cell signaling by endocannabinoids and their congeners: questions of selectivity and other challenges. *Chemistry and Physics of Lipids* 2002; **121**:111-134.

Schwarz H, Blanco F, Lotz M. Anadamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis. *Journal of Neuroimmunology* 1994; **55**:107-115.

Shire D, Calandra B, Bouaboula M, Barth F, Rinaldi-Carmona M, Casellas P, Ferrara P. Cannabinoid receptor interactions with the antagonists SR141716A and SR144528. *Life Sciences* 1999; **65**:627-635.

Shire D, Carillon C, Kaghad M, Calandra B, Rinaldi-Carmona M, Le Fur G, Caput D, Ferrara P. An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. *Journal of Biological Chemistry* 1995; **270**:3726-3731.

Shoemaker J, Joseph B, Ruckle M, Mayeux P, Prather P. The endocannabinoid noladin ether acts as a full agonist at human CB2 cannabinoid receptors. *Journal of Pharmacology and Experimental Therapeutics* 2005a; **314**:868-875.

Shoemaker J, Ruckle M, Mayeux P, Prather P. Agonist-directed trafficking of response by endocannabinoids acting at CB2 receptors. *Journal of Pharmacology and Experimental Therapeutics* 2005b; **315**:828-838.

Slipetz D, O'Neill G, Favreau L, Dufresne C, Gallant M, Gareau Y, Guay D, Labelle M, Metters K. Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. *Molecular Pharmacology* 1995; **48**:352-361.

Smart D, Gunthorpe M, Jerman J, Nasir S, Gray J, Muir A, Chambers J, Randall A, Davis J. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *British Journal of Pharmacology* 2000; **129**:227-230.

Smith S, Denhardt G, Terminelli C. The anti-inflammatory activities of cannabinoid receptor ligands in mouse peritonitis models. *European Journal of Pharmacology* 2001; **432**:107-119.

Song C, Howlett A. Rat brain cannabinoid receptors are *N*-linked glycosylated proteins. *Life Sciences* 1995; **56**:1983-1989.

Song G, Ouyang G, Bao S. The activation of AKT/PKB signaling pathway and cell survival. *Journal of Cellular and Molecular Medicine* 2005; **9**:59-71.

Sotsios Y, Whittaker G, Westwick J, Ward S. The CXC chemokine stromal cell-derived factor activates a G(i)-coupled phosphoinositide 3-kinase in T lymphocytes. *Journal of Immunology* 1999; **163**:5954-5963.

Srivastava M, Srivastava B, Brouhard B. Δ^9-Tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. *Immunopharmacology* 1998; **40**:179-185.

Steeber D, Venturi G, Tedder T. A new twist to the leukocyte adhesion cascade: intimate cooperation is key. *Trends in Immunology* 2005; **26**:9-12.

Steele A, Szabo I, Bednar F, Rogers T. Interactions between opioid and chemokine receptors: heterologous desensitization. *Cytokine & Growth Factor Reviews* 2002; **13**:209-222.

Steffens M, Zentner J, Honegger J, Feuerstein T. Binding affinity and agonist activity of putative endogenous cannabinoids at the human neocortical CB1 receptor. *Biochemical Pharmacology* 2005a; **69**:169-178.

Steffens S, Veillard N, Arnaud C, Pelli G, Burger F, Staub C, Zimmer A, Frossard J, Mach F. Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. *Nature* 2005b; **434**:782-786.

Stein J, Nombela-Arrieta C. Chemokine control of lymphocyte trafficking: a general overview. *Immunology* 2005; **116**:1-12.

Stella N, Schweitzer P, Piomelli D. A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 1997; **388**:773-778.

Stork P. Does Rap1 deserve a bad Rap? *Trends in Biochemical Sciences* 2003; **28**:267-275.

Stork P, Schmitt J. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends in Cell Biology* 2002; **12**:258-266.

Sugiura T, Kobayashi Y, Oka S, Waku K. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukotrienes and Essentential Fatty Acids* 2002; **66**:173-192.

Sugiura T, Kodaka T, Kondo S, Tonegawa T, Nakane S, Kishimoto S, Yamashita A, Waku K. 2-Arachidonoylglycerol, a putative endogenous cannabinoid receptor ligand, induces rapid, transient elevation of intracellular free Ca^{2+} in neuroblastoma x glioma hybrid NG108-15 cells. *Biochemical and Biophysical Research Communications* 1996; **229**:58-64.

Sugiura T, Kodaka T, Nakane S, Miyashita T, Kondo S, Suhara Y, Takayama H, Waku K, Seyi C, Baba N, Ishima Y. Evidence that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. Structure-activity relationship of 2-arachidonoylglycerol, ether-linked analogues, and related compounds. *Journal of Biological Chemistry* 1999; **274**:2794-2801.

Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, Suhara Y, Takayama H, Waku K. Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *Journal of Biological Chemistry* 2000; **275**:605-612.

Suire S, Coadwell J, Ferguson G, Davidson K, Hawkins P, Stephens L. p84, a new Gβγ-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110γ. *Current Biology* 2005; **15**:566-570.

Sun Y, Tsuboi K, Zhao L, Okamoto Y, Lambert D, Ueda N. Involvement of *N*-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other *N*-acylethanolamines in macrophages. *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids* 2005; **1736**:211-220.

Sunahara R, Taussig R. Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. *Molecular Interventions* 2002; **2**:168-184.

Tan S, Parker P. Emerging and diverse roles of protein kinase C in immune cell signalling. *Biochemical Journal* 2003; **376**:545-552.

Terrillon S, Bouvier M. Roles of G-protein-coupled receptor dimerization. *EMBO Reports* 2004; **5**:30-34.

Thomas A, Stevenson L, Wease K, Price M, Baillie G, Ross R, Pertwee R. Evidence that the plant cannabinoid Δ^9-tetrahydrocannabivarin is a cannabinoid CB1 and CB2 receptor antagonist. *British Journal of Pharmacology* 2005; **146**:917-926.

Thompson C, Powrie F. Regulatory T cells. *Current Opinion in Pharmacology* 2004; **4**:408-414.

Toth A, Kedei N, Wang Y, Blumberg P. Arachidonyl dopamine as a ligand for the vanilloid receptor VR1 of the rat. *Life Sciences* 2003; **73**:487-498.

Tsuboi K, Sun Y, Okamoto Y, Araki N, Tonai T, Ueda N. Molecular characterization of *N*-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. *Journal of Biological Chemistry* 2005; **280**:11082-11092.

Ueda N, Yamanaka K, Yamamoto S. Purification and characterization of an acid amidase selective for *N*-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. *Journal of Biological Chemistry* 2001; **276**:35552-35557.

Ueda N, Yamanaka K, Terasawa Y, Yamamoto S. An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. *FEBS Letters* 1999; **454**:267-270.

Valjent E, Pages C, Rogard M, Besson M, Maldonado R, Caboche J. Δ^9-Tetrahydrocannabinol-induced MAPK/ERK and Elk-1 activation *in vivo* depends on dopaminergic transmission. *European Journal of Neuroscience* 2001; **14**:342-352.

Valk P, Verbakel S, Vankan Y, Hol S, Mancham S, Ploemacher R, Mayen A, Lowenberg B, Delwel R. Anandamide, a natural ligand for the peripheral cannabinoid receptor is a novel synergistic growth factor for hematopoietic cells. *Blood* 1997; **90**:1448-1457.

Van der Stelt M, Di Marzo V. Anandamide as an intracellular messenger regulating ion channel activity. *Prostaglandins and Other Lipid Mediators* 2005; **77**:111-122.

Van Haastert P, Devreotes P. Chemotaxis: signalling the way forward. *Nature Reviews in Molecular Cell Biology* 2004; **5**:626-634.

Van Sickle, Duncan M, Kingsley P, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison J, Marnett L, Di Marzo V, Pittman Q, Patel K, Sharkey K. Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 2005; **310**:329-332.

Vanhaesebroeck B, Alessi D. The P13K-PDK1 connection: more than just a road to PKB. *Biochemical Journal* 2000; **346**:561-576.

Vasquez C, Lewis D. The CB1 cannabinoid receptor can sequester G-proteins, making them unavailable to couple to other receptors. *Journal of Neuroscience* 1999; **19**:9271-9280.

Viatour P, Merville M, Bours V, Chariot A. Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation. *Trends in Biochemical Sciences* 2005; **30**:43-52.

Vicente-Manzanares M, Webb D, Horwitz A. Cell migration at a glance. *Journal of Cell Science* 2005; **118**:4917-4919.

Villalba M, Kasibhatla S, Genestier L, Mahboubi A, Green D, Altman A. Protein kinase Cθ cooperates with calcineurin to induce Fas ligand expression during activation-induced T cell death. *Journal of Immunology* 1999; **163**:5813-5819.

Vlahos C, Matter W, Hui K, Brown R. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *Journal of Biological Chemistry* 1994; **269**:5241-5248.

Wager-Miller J, Westenbroek R, Mackie K. Dimerization of G protein-coupled receptors: CB1 cannabinoid receptors as an example. *Chemistry and Physics of Lipids* 2002; **121**:83-89.

Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, Mackie K, Stella N. Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *Journal of Neuroscience* 2003; **23**:1398-1405.

Ward S, Sotsios Y, Dowden J, Bruce I, Finan P. Therapeutic potential of phosphoinositide 3-kinase inhibitors. *Chemistry & Biology* 2003; **10**:207-213.

Wartmann M, Campbell D, Subramanian A, Burstein S, Davis R. The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Letters* 1995; **359**:133-136.

Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* 2003; **424**:434-438.

Way K, Chou E, King G. Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends in Pharmacological Sciences* 2000; **21**:181-187.

Werry T, Sexton P, Christopoulos A. "Ins and outs" of seven-transmembrane receptor signalling to ERK. *Trends in Endocrinology and Metabolism* 2005; **16**:26-33.

Wiley J, Martin B. Cannabinoid pharmacology: implications for additional cannabinoid receptor subtypes. *Chemistry and Physics of Lipids* 2002; **121**:57-63.

Wilkinson S, Parker P, Nixon J. Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochemical Journal* 1993; **294**:335-337.

Woodgett J. Recent advances in the protein kinase B signaling pathway. *Current Opinion in Cell Biology* 2005; **17**:150-157.

Wright K, Rooney N, Feeney M, Tate J, Robertson D, Welham M, Ward S. Differential expression of cannabinoid receptors in the human colon: Cannabinoids promote epithelial wound healing. *Gastroenterology* 2005; **129**:437-453.

Xie X, Chen J, Billings E. 3D structural model of the G-protein-coupled cannabinoid CB2 receptor. *Proteins* 2003; **53**:307-319.

Xu W, Filppula S, Mercier R, Yaddanapudis S, Pavlopoulos S, Cai J, Pierce W, Makriyannis A. Purification and mass spectroscopic analysis of human CB1 cannabinoid receptor functionally expressed using the baculovirus system. *Journal of Peptide Research* 2005; **66**:138-150.

Yuan M, Kiertscher S, Cheng Q, Zoumalan R, Tashkin D, Roth M. Δ^9-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T cells. *Journal of Neuroimmunology* 2002; **133**:124-131.

Zhang W, Samelson L. The role of membrane-associated adaptors in T cell receptor signalling. *Seminars in Immunology* 2000; **12**:35-41.

Zhao Q, He Z, Chen N, Cho Y-Y, Zhu F, Lu C, Ma W, Bode A, Dong Z. 2-Arachidonoylglycerol stimulates activator protein-1-dependent transcriptional activity and enhances epidermal growth factor-induced cell transformation in JB6 P+ cells. *Journal of Biological Chemistry* 2005; **280**:26735-26742.

Zhou D, Song Z. CB1 cannabinoid receptor-mediated tyrosine phosphorylation of focal adhesion kinase-related non-kinase. *FEBS Letters* 2002; **525**:164-168.

Zhu W, Friedman H, Klein TW. Δ^9-Tetrahydrocannabinol induces apoptosis in macrophages and lymphocytes: Involvement of Bcl-2 and caspase-1. *Journal of Pharmacology and Experimental Therapeutics* 1998; **286**:1103-1109.

Zoratti C, Kipmen-Korgun D, Osibow K, Malli R, Graier W. Anandamide initiates Ca^{2+} signaling via CB2 receptor linked to phospholipase C in calf pulmonary endothelial cells. *British Journal of Pharmacology* 2003; **140**:1351-1362.

Zygmunt P, Petersson J, Andersson D, Chuang H, Sorgard M, Di Marzo V, Julius D, Hogestatt E. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 1999; **400**:452-457.

Chapter 7: Appendices

7.1 Additional details on methods

7.1.1 Recipes for solutions/buffers used in immunoblotting

7.1.1.1 Lysis buffers

Lysis buffer

50mM Tris pH 7.5
150mM NaCl
1% (v/v) Nonidet P-40
10% (v/v) glycerol
5mM EDTA
1mM sodium vanadate *
1mM sodium molybdate *
10mM sodium fluoride *
40µg/ml phenylmethylsulfonyl fluoride *
0.7µg/ml pepstatin A *
10µg/ml aprotinin *
10µg/ml leupeptin *
10µg/ml soybean trypsin inhibitor *
MilliQ H_2O

Hypotonic lysis buffer

10mM HEPES pH 7.2
5mM EDTA
1mM sodium vanadate *
1mM sodium molybdate *
10mM sodium fluoride *
40µg/ml phenylmethylsulfonyl fluoride *
0.7µg/ml pepstatin A *
10µg/ml aprotinin *
10µg/ml leupeptin *
10µg/ml soybean trypsin inhibitor *
MilliQ H_2O

* added just before use

7.1.1.2 5x SDS-sample buffer

10% (w/v) SDS
50% (v/v) glycerol
200mM Tris pH 6.8
Bromophenol blue
5% (v/v) 2-mercaptoethanol
MilliQ H_2O

7.1.1.3 Stacking & resolving gels

To make 4 gels:

	Resolving		Stacking
	10%	**12%**	**5%**
Acrylamide	6.67ml	8ml	1.67ml
MilliQ H_2O	5.8ml	4.5ml	6ml
1M Tris	7.5ml (pH 8.8)	7.5ml (pH 8.8)	1.25ml (pH 6.8)
10% (w/v) SDS	0.33ml	0.33ml	0.15ml
10% (w/v) ammonium persulphate *	66.7µl	66.7µl	50µl
TEMED *	26.7µl	26.7µl	20µl

* added just before use

7.1.1.4 Buffers for immunoblotting

SDS-PAGE running buffer

25mM Tris

192mM glycine

0.1% (w/v) SDS

MilliQ H_2O

Semi-dry transfer buffer

39mM glycine

48mM Tris

0.0375 % (w/v) SDS

20% (v/v) methanol

MilliQ H_2O

Tris-buffered saline (TBS)

20mM Tris pH7.5

150mM NaCl

MilliQ H_2O

Stripping buffer

62.5mM Tris pH6.8

2% (w/v) SDS

0.0077% (v/v) 2-mercaptoethanol

MilliQ H_2O

TBS–tween (TBST)

TBS as above

0.01% (v/v) Tween-20

7.1.2 Contidions for antibodies used in immunoblotting

Antibody	Block (in TBS)	Primary antibody dilution (in TBS + 0.01% sodium azide)	Secondary antibody dilution (in TBST)
CB_1R (Affinity Bioreagents)	5% BSA	1:1000	Anti-rabbit, 1:7000 1% marvel
CB_1R (Cayman Chemicals)	5% marvel	1:1000	Anti-rabbit, 1:7000 1% marvel
CB_2R (Cayman Chemicals)	5% marvel	1:500	Anti-rabbit, 1:7000 1% marvel
CB_1R (Santa Cruz)	5% marvel	1:1000 1% BSA	Anti-rabbit, 1:10000 2% marvel
CB_2R (Santa Cruz)	5% marvel	1:1000 1% BSA	Anti-rabbit, 1:10000 2% marvel
FAAH	5% BSA	1:500	Anti-rabbit 1:7000 1% marvel
IκBα	5% marvel	1:1000	Anti-rabbit, 1:10000
MAGL	5% BSA	1:1000	Anti-rabbit, 1:7000 1% marvel
Pan ERK	5% marvel	1:1000	Anti-rabbit, 1:10000
Pan PKB	5% marvel	1:1000	Anti-goat, 1:10000
Phospho-ERK1/2$^{Thr202/Tyr204}$	5% marvel	1:1000	Anti-rabbit, 1:10000
Phospho-PKB^{Ser473}	1% marvel	1:1000	Anti-rabbit, 1:7000 1% marvel

7.2 Details on MEK, PKC and PI3K inhibitors used in *Results II*

7.2.1 IC_{50}s

Compound	Kinases affected	IC_{50} (μM)	References
Gö6976	PKCα	0.0023	Martiny-Baron *et al.*, 1993
	PKCβI	0.0062	
	PKCδ	no effect	
	PKCε	no effect	
	PKCζ	no effect	
LY294002	PI3K	1.4	Vlahos *et al.*, 1994
	DNA-PK	0.66	Knight *et al.*, 2004
	MTOR	8.9	
	Casein kinase II	12.2	
PD98059	MEK1	2-7	Alessi *et al.*, 1995
Ro-32-0432	PKCα	0.009	Wilkinson *et al.*, 1993
	PKCβI	0.028	
	PKCβII	0.031	
	PKCγ	0.037	
	PKCε	0.108	
Rottlerin	PKCα	30	Gschwendt *et al.*, 1994
	PKCβ	42	
	PKCγ	40	
	PKCδ	3-6	
	PKCε	100	
	PKCζ	100	
	PKCη	82	
	PKCθ	>100	Villalba *et al.*, 1999
	Casein kinase II	30	Gschwendt *et al.*, 1994
	PKA	78	
	CaM kinase III	5.3	
	PRAK	1.9	Davies *et al.*, 2000
	MAPKAP-K2	5.4	

7.2.2 Chemical names

Compound	Chemical name
Gö6976	12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole
LY294002	2-(-4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
Ro-32-0432	Bisindoylmaleimide XI; 2-(8-[dimethylamino}methyl]-6,7,8,9-tetrahydropyroido[1,2-a]indol-3-yl)-3-(1-methylindol-3-yl)maleimide
Rottlerin	3'-[(8-Cinnamoyl-5,7-dihydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-yl)methyl]-2',4',6'-trihydroxy-5'-methylacetophenone

7.3 Chemical names of cannabinergic ligands used in this study

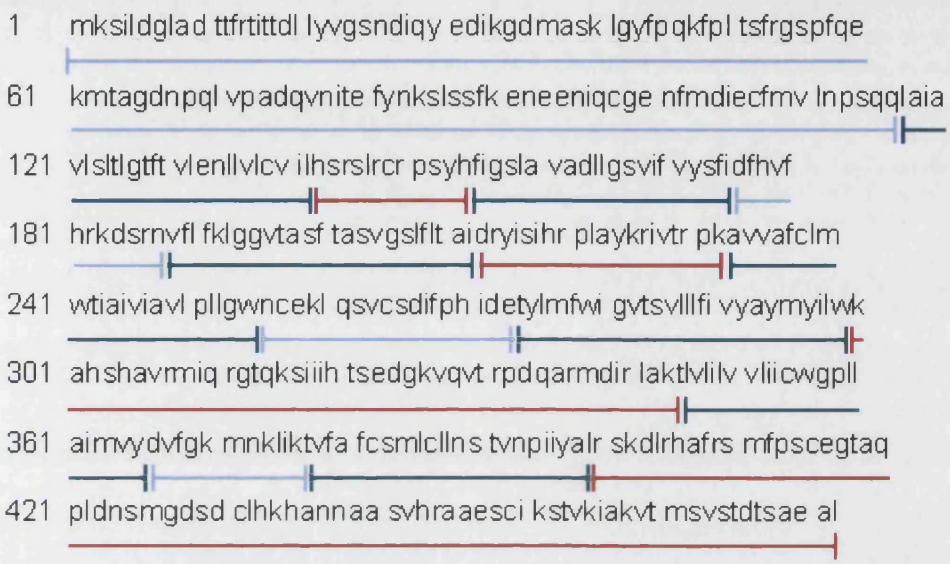
Cannabinergic ligand	Chemical name
2-AG	(5Z,8Z,11Z,14Z)-5,8,11,14-Eicosatetraenoic acid, 2-hydroxy-1-(hydroxomethyl)ethyl ester
ACPA	*N*-(2-Cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide
AM251	*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide
AM630	6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone
AEA	Arachidonoylethanolamide; *N*-(2-Hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide
JWH-133	(6a*R*,10a*R*)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9,-trimethyl-6*H*-dibenyo[b,d]pyran
MAFP	Methyl arachidonoyl fluorophosphonate; (5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenyl-methyl ester phosphonofluoridic acid
Met-AEA	(*R*)-*N*-(2-Hydroxy-1-methylethyl)-5Z,8Z,11Z,14Z-eicoatetraenamide

7.4 Details on cannabinoid receptors

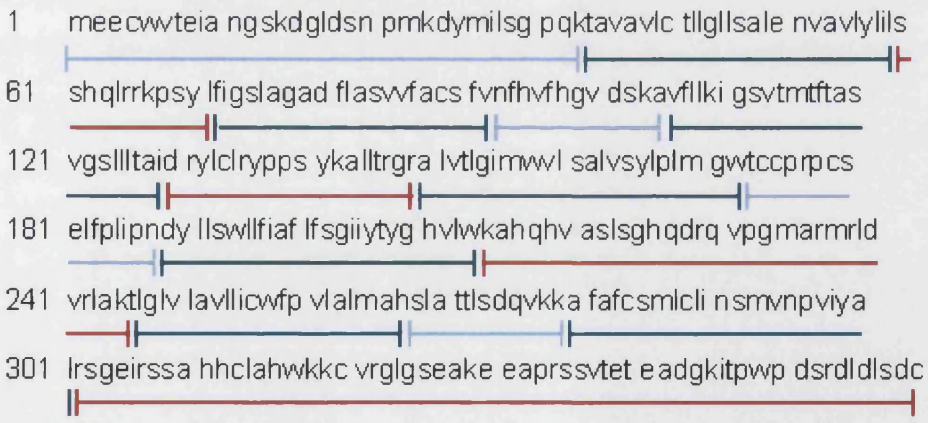
7.4.1 Cannabinoid receptor amino acid sequences

Below are the amino acid sequences for the human CB_1R and CB_2R. The potential extracellular (—), transmembrane (—) and intracellular (—) domains are marked.

CB_1R (human) amino acid sequence:



CB_2R (human) amino acid sequence:



7.4.2 Cannabinoid receptor antibodies used

Antibody	Source	Raised against
CB_1R	Cayman Chemicals	Amino acids 1-14 of human CB_1R
CB_1R	Santa Cruz	Amino acids 1-150 of the human CB_1R
CB_1R	Affinity Bioreagents	Amino acids 1-99 of the human CB_1R
CB_2R	Cayman Chemicals	Amino acids 20-33 of the human CB_2R
CB_2R	Santa Cruz	Amino acids 301-360 of the human CB_2R

7.5 Amino Acid Codes

Single letter code	3 letter code	Amino Acid
A	Ala	Alanine
B	Asx	Asparagine/Aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine/Glutamic acid